

20-July-2006



---

Betaine Homocysteine Methyltransferase, Disease and Diet

The Use of Proton Nuclear Magnetic Resonance  
on Biological Methylamines

A thesis submitted in partial fulfilment of the requirements  
for the degree of

Doctor of Philosophy

in the University of Canterbury

by

Martin Bryce Lee

# Table of Contents

Table of Figures.....	v
Acknowledgements .....	vi
Abstract.....	vii
Table of Publications Resulting From This Thesis .....	viii
Table of Abbreviations and Symbols.....	ix
<b>CHAPTER 1: Introduction .....</b>	<b>2</b>
<b>1.1 Cardiovascular Disease.....</b>	<b>2</b>
1.1.1 The CVD Epidemic.....	2
1.1.2 Causes of CVD .....	3
<b>1.2 Homocysteine and Glycine Betaine .....</b>	<b>4</b>
1.2.1 Homocysteine Biochemistry .....	4
1.2.2 Betaines .....	6
1.2.3 Diet and Homocysteine .....	7
1.2.4 Betaine Metabolism .....	9
1.2.5 Homocysteine Disorders .....	10
<b>1.3 Betaine Homocysteine Methyltransferase .....</b>	<b>11</b>
1.3.1 BHMT .....	11
1.3.2 BHMT Mechanism.....	14
1.3.3 BHMT Distribution.....	17
1.3.4 Methionine Synthase.....	18
1.3.5 BHMT Genetics .....	20
1.3.6 BHMT Regulation.....	21
<b>1.4 Assays for BHMT.....</b>	<b>25</b>
1.4.1 BHMT Assays .....	25
1.4.2 Radiochemical Assays .....	25
1.4.3 HPLC Methods .....	25
1.4.4 <i>In Vivo</i> Bacterial Assay .....	26
1.4.5 Nuclear Magnetic Resonance (NMR) Assays .....	27
<b>1.5 Thesis Aims .....</b>	<b>28</b>
References .....	29
<b>CHAPTER 2: Protein Purification .....</b>	<b>37</b>
<b>2.1 Introduction .....</b>	<b>37</b>
<b>2.2 Sources of Protein.....</b>	<b>38</b>
<b>2.3 Heat Denaturation .....</b>	<b>39</b>
2.3.1 Introduction .....	39
2.3.2 Method and Results .....	39
<b>2.4 Anion-Exchange Chromatography .....</b>	<b>40</b>
2.4.1 Introduction .....	40
2.4.2 Method.....	41
2.4.3 Results.....	41

<b>2.5 Size Exclusion Chromatography.....</b>	<b>42</b>
2.5.1 Introduction .....	42
2.5.2 Method.....	43
2.5.3 Results.....	44
<b>2.6 Combined strategy.....</b>	<b>45</b>
2.6.1 Introduction .....	45
2.6.2 Final Method .....	46
2.6.3 Results.....	48
<b>2.7 Mass Spectrometry Analysis.....</b>	<b>49</b>
2.7.1 Introduction .....	49
2.7.2 Method.....	50
2.7.3 Results.....	51
<b>2.8 N-terminal Sequencing.....</b>	<b>54</b>
2.8.1 Introduction .....	54
2.8.2 Method.....	54
2.8.3 Results.....	54
<b>2.9 Genomic Sequencing of Rat <i>BHMT</i>.....</b>	<b>55</b>
2.9.1 Introduction .....	55
2.9.2 Method.....	55
2.9.3 Results.....	57
<b>2.10 Discussion .....</b>	<b>59</b>
<b>2.11 Conclusion .....</b>	<b>62</b>
<b>References .....</b>	<b>63</b>
<b>CHAPTER 3: NMR Spectroscopic Assays.....</b>	<b>65</b>
<b>3.1 NMR Concepts.....</b>	<b>65</b>
3.1.1 Introduction .....	65
3.1.2 Nuclear Magnetic Resonance .....	66
3.1.3 Spin, and the Nuclear Magnetic Resonance Effect.....	66
3.1.4 Pulse Sequences .....	70
<b>3.2 Quantitative NMR Spectroscopy .....</b>	<b>71</b>
3.2.1 Introduction .....	71
3.2.2 $T_1$ Relaxation.....	71
3.2.3 $T_2$ Relaxation.....	73
3.2.4 Internal Standards for Quantification .....	74
3.2.5 Aqueous Measurements.....	75
<b>3.3 Relaxation Measurements of Methylamines.....</b>	<b>76</b>
3.3.1 Introduction .....	76
3.3.2 Method.....	76
3.3.3 Results.....	77
<b>3.4 Validation of <math>^1\text{H}</math>-NMR Spectroscopy for Urine Methylamine Measurements .....</b>	<b>77</b>
3.4.1 Introduction .....	77
3.4.2 Method.....	78
3.4.3 Results.....	80
<b>3.5 Discussion .....</b>	<b>85</b>
<b>3.6 Conclusion .....</b>	<b>87</b>
<b>References .....</b>	<b>88</b>

<b>CHAPTER 4: Kinetic Measurements of BHMT .....</b>	<b>94</b>
4.1 Introduction .....	94
4.2 Kinetics of BHMT with Glycine Betaine .....	96
4.2.1 Introduction .....	96
4.2.2 Materials and Methods .....	97
4.2.3 Results.....	99
4.3 The Endpoint Assay.....	103
4.3.1 Introduction .....	103
4.3.2 Method.....	104
4.3.3 Results.....	104
4.4 Discussion .....	105
4.5 Conclusion .....	108
References .....	109
<b>CHAPTER 5: Alternative Methyl Donors with BHMT .....</b>	<b>111</b>
5.1 Methyl Donors in BHMT .....	111
5.1.1 Introduction .....	116
5.1.2 Method.....	117
5.1.3 Results.....	120
5.1.3.1 Activity Studies .....	120
5.1.3.2 Inhibition/Allosteric Effects.....	125
5.2 Discussion .....	131
5.3 Conclusion .....	139
References .....	141
<b>CHAPTER 6: Comparative Studies of BHMT from Mammals, Agnathans and Other Species .....</b>	<b>145</b>
6.1 Introduction .....	145
6.2 Activity Studies.....	147
6.2.1 BHMT Activity in Lampreys .....	147
6.2.2 Method.....	148
6.2.3 Results.....	150
6.3 Purification of Hagfish BHMT .....	151
6.3.1 Introduction .....	151
6.3.2 Method.....	151
6.3.2 Results.....	152
6.4 <i>N</i> -terminal Sequencing.....	152
6.4.1 Introduction .....	152
6.4.2 Method.....	152
6.4.3 Results.....	153
6.5 Comparison of Rat and Hagfish BHMT Activity .....	156
6.5.1 Background to Comparison Studies .....	156
6.5.2 Method.....	157
6.5.3 Results.....	158
6.6 Discussion .....	161
6.7 Conclusion .....	163

References .....	165
<b>CHAPTER 7: <i>In Vivo</i> Studies of Mammalian Betaine Metabolism.....</b>	<b>170</b>
<b>7.1 Introduction .....</b>	<b>170</b>
7.1.1 Methylation <i>in Vivo</i> .....	170
7.1.2 Whole Animal Studies .....	172
<b>7.2 Rat Models of Betaine Metabolism .....</b>	<b>174</b>
7.2.1 Betaine Homeostasis .....	174
7.2.2 Study design .....	174
7.2.3 Results.....	175
7.2.4 Discussion.....	181
<b>7.3 Chronic Renal Failure in Sheep.....</b>	<b>183</b>
7.3.1 Renal Failure and Homocysteine .....	183
7.3.2 Study Design .....	183
7.3.3 Results.....	184
7.3.4 Discussion.....	185
<b>7.4 Coffee and Trigonelline .....</b>	<b>187</b>
7.4.1 Coffee Consumption and Homocysteine.....	187
7.4.2 Study Design .....	187
7.4.3 Results.....	188
7.4.4 Discussion.....	191
<b>7.5 Dimethylglycine (DMG) Supplementation .....</b>	<b>193</b>
7.5.1 Dimethylglycine and Methylation .....	193
7.5.2 Study Design .....	194
7.5.3 Results.....	195
7.5.4 Discussion.....	195
References .....	197
<b>CHAPTER 8: Conclusions and Future Work .....</b>	<b>201</b>
8.1 Thesis Aims .....	201
8.2 Reproducible BHMT Purification .....	201
8.3 <sup>1</sup> H-NMR Spectroscopic Assay for BHMT and Kinetic Measurements .....	204
8.4 Methyl Donor Substrate Specificity .....	205
8.5 Comparison of Agnathan and Mammalian Liver BHMT.....	206
8.6 <sup>1</sup> H-NMR Spectroscopic Assay for Methylamines in Urine .....	208
8.7 Future Experiments .....	210
8.8 Conclusion .....	211
References .....	212
<b>Appendix 1 Tryptic Digest of BHMT .....</b>	<b>215</b>

## Table of Figures

Figure	Description	Page
Figure 1.1	Death rate and CVD for selected countries	3
Figure 1.2	Homocysteine metabolism (abridged)	5
Figure 1.3	Betaines used in this thesis	6
Figure 1.4	Oxidation of choline to produce methylene tetrahydrofolate	8
Figure 1.5	Evans <i>et al</i> /crystal structure of recombinant BHMT	12
Figure 1.6	Gonzalez <i>et al</i> /structure of rat liver recombinant BHMT	13
Figure 1.7	Transition state inhibitor of BHMT	14
Figure 1.8	Schematic of an ordered bi-bi mechanism	16
Figure 1.9	BHMT mechanism as determined by IF spectroscopy	16
Figure 1.10	Methionine synthase reactions	18
Figure 2.1	Temperature dependence and time course of heat denaturation	40
Figure 2.2	Calibration of G-200 column	44
Figure 2.3	G-200 elution trace, with inset of BHMT activity	45
Figure 2.4	SDS-PAGE analysis of rat liver purification	49
Figure 2.5	Predicted rat BHMT amino acid sequence with tryptic fragments	51
Figure 2.6	Mass spectrum of tryptic digest fragments	53
Figure 2.7	Wistar rat BHMT genomic DNA sequence	56
Figure 2.8	PCR products analysed by 2% agarose gel	57
Figure 2.9	Alignment of experimentally determined BHMT sequences	58
Figure 3.1	The bulk magnetisation vector	68
Figure 3.2	Bulk magnetisation vector after a 90 ° pulse	69
Figure 3.3	An 'inversion recovery' experiment pulse sequence	72
Figure 3.4	Example of 'inversion recovery' result	73
Figure 3.5	Relaxation from transverse relaxation	74
Figure 3.6	Human urine <sup>1</sup> H-NMR spectrum showing metabolites of interest	81
Figure 3.7	Standard lines for the metabolites of interest	83
Figure 4.1	<sup>1</sup> H-NMR spectrum from kinetic analysis	100
Figure 4.2	Layered sequential <sup>1</sup> H-NMR spectra showing BHMT activity	101
Figure 4.3	BHMT activity, as monitored by <sup>1</sup> H-NMR spectroscopy	102
Figure 4.4	Lineweaver-Burk plot of BHMT activity	103
Figure 4.5	Standard line for endpoint assay	105
Figure 6.1	Hagfish BHMT PVDF blot	154
Figure 6.2	Alignment of several BHMT amino acid sequences	155
Figure 7.1	Plasma homocysteine following betaine administration in rats	176
Figure 7.2	Plasma concentrations of administered betaines	177
Figure 7.3	Changes in plasma betaine and DMG after betaine administration	179
Figure 7.4	Rat urine following DMT treatment	181
Figure 7.5	Plasma homocysteine post coffee and trigonelline administration	190
Figure 7.6	Urine excretion of glycine betaine after treatments	190

## Acknowledgements

I have come to the conclusion that the process of completing a body of work like a Ph.D. thesis is less a solitary pursuit than a collaborative effort combining an impressive support team. Certainly, my work would have been utterly impossible without an entourage of friends, family and colleagues.

The first point of call for all my research worries, was my team of supervisors, Professors John Blunt, Peter George, Steve Brennan and Steve Chambers, as well as Dr Michael Lever. They have all been supportive, encouraging, wise and knowledgeable when any problems have arisen from my work.

I have been surrounded by a tolerant and sympathetic group of students and co-researchers at both Canterbury Health Laboratories (Sarah Molyneux, Malina Storer, Dr Wendy Atkinson, Madhusudan Vasudevamurthy and Chris M'Entyre) and the University of Canterbury (Anna McCarthy, Marie Squire, Sarah Lundy, Andrew Muscroft-Taylor, Jonno Hill, Liesl Marsh, Dan Comeski, Dr Jan Wikaira and Rebecca Hurrell). I have been fortunate enough to have been adopted by the last members of the Morris Synthetic Group, who have been an endless source of fun to work with. Special note should be made for the Gerrard group in the School of Biological Sciences at the University of Canterbury, who have taken me under their wing, and shared equipment and help.

During the course of this work, I have been hosted in Sydney by Prof Philip Kuchel, and in Champaign/Urbana by Prof Tim Garrow. I am grateful for their hospitality, and for the useful discussions that I have been able to have with both these people. Dr Bill Bubbs at the University of Sydney was also a source of encyclopaedic discussions during my second year of work. I'm very thankful to all of them for this help.

An extra large "thanks" must go to Dr Sandy Slow, who has been a far larger influence on my research than either her, or I, could have predicted. Her long distance support has been unwavering and I thank her for every second of it.

My family deserve Ph.D.'s of their own (psychology?) for their support and understanding. I'm blessed with a family who are always keen to at least pretend to know what I am doing, and are tolerant of the mood swings theses induce. Mary, Peter, Simon, and Johnny Lee have all been a big part of this work.

I am also blessed with an extended network of friends beyond the ivory towers of the University, who would all deserve a mention here. Craig Seagar and Ross Palmer, who have always been available with alcohol to sooth the ailing student, Ali Adams, one of the most amazing women on earth who has mentored me, and Jane Brennan, who has been a stable shoulder to cry on, all deserve mention.

Financial support has also been provided by the New Zealand Health Research Council (HRC), the National Heart Foundation (NHF), the Foundation for Research, Science and Technology (FRST) and the Tertiary Education Council (TEC).

Finally, and most importantly, is my partner in life, Clodoaldo Martins, who has supported, understood, encouraged and helped at all of the important times. He has been the one who has suffered the most as I have been persevering. Through it all, he was with me.

## Abstract

Homocysteine, an independent risk factor for cardiovascular disease, is methylated in the liver via the zinc metalloenzyme betaine-homocysteine methyltransferase (BHMT). Established assays for BHMT include a radiochemical assay, a colorimetric assay, an HPLC assay and an *in vivo* microbiological assay. These techniques are either unsuitable for substrate specificity studies, or are unable to give kinetic measurements. BHMT was purified from liver and measured directly and kinetically by a novel  $^1\text{H}$ -NMR spectroscopic assay. The disappearance of substrates and the formation of products are monitored simultaneously.

Using 2 mM glycine betaine and homocysteine as substrates in 20 mM phosphate buffer (pH = 7.5) and measuring the production of N,N-dimethylglycine the CV is 6.3% (n = 6) and the detection limit is 6 nkatal. An endpoint assay for BHMT activity was also developed and had CV = 5.3%, n = 6, with a detection limit of 2 nkatal. The NMR spectroscopic assay was used to determine the substrate specificity with a library of alternative substrates. Analysis of betaine analogues with different chain length,  $\alpha$ -substitution, substitution of the nitrogen and carboxyl moieties demonstrated that BHMT is inactive if there is any steric crowding of the nitrogen or  $\alpha$ -carbon positions. BHMT is capable of using group VI heteroatom betaines as methyl donors, with much faster rates than glycine betaine. For glycine betaine the  $K_m$  was  $0.19 \pm 0.03$  mM with a  $V_{max}$  of  $17 \pm 0.7$  nMol min<sup>-1</sup> mg<sup>-1</sup>. The same assay was used to detect and partially characterise a BHMT activity from hagfish liver that is similar to that of the mammalian enzyme.

NMR spectroscopy was adapted for measurements of glycine betaine in urine, along with other medically significant methylamines. These were shown to be valid for clinical use and in animal studies. A novel metabolite of the sulfonium analogue of glycine betaine (methylsulfinylmethanoate) was identified in rats.



## Table of Publications Resulting From This Thesis

### Papers:

**Lee, M. B.;** Blunt, J. W.; Lever, M.; George, P. M. A nuclear-magnetic-resonance-based assay for betaine-homocysteine methyltransferase activity: *Analytical Biochemistry* **2004**, *330*, 199-205.

Slow, S.; Lever, M.; **Lee, M. B.;** George, P. M.; Chambers, S. T. Betaine analogues alter homocysteine metabolism in rats: *International Journal of Biochemistry & Cell Biology* **2004**, *36*, 870-880.

Slow, S.; Miller, W. E.; McGregor, D. O.; **Lee, M. B.;** Lever, M.; George, P. M.; Chambers, S. T. Trigonelline is not responsible for the acute increase in plasma homocysteine following ingestion of instant coffee: *European Journal of Clinical Nutrition* **2004**, *58*, 1253-1256.

Slow, S.; McGregor, D. O.; Lever, M.; **Lee, M. B.;** George, P. M.; Chambers, S. T. Dimethylglycine supplementation does not affect plasma homocysteine concentrations in pre-dialysis chronic renal failure patients: *Clinical Biochemistry* **2004**, *37*, 974-976.

**Lee, M. B.;** Storer, M. K.; Blunt, J. W.; Lever, M. Validation of <sup>1</sup>H NMR spectroscopy as an analytical tool for methylamine metabolites in urine: *Clinica Chimica Acta* **2006**, *365*, 264-269.

**Lee, M. B.;** Chambers, S.T.; George, P. M.; Brennan, S. T O. Hematopoietic antimicrobial peptide-37, identification of the protein and activity from Pacific Hagfish: Comparative Biochemistry and Physiology, In Preparation.

**Lee, M.B.;** Chambers, S.T.; George, P.M.; Lever, M.; Blunt, J.; MacLagan, R. G. A. R. Substrate specificity and inhibition of rat liver BHMT: In Preparation.

**Lee, M.B.;** Chambers, S.T.; George, P.M.; Lever, M. Comparison of hagfish and rat BHMT activities: In Preparation.

### Poster Presentations:

"An NMR Based Assay For BHMT" Presented to MedSci2004, Queenstown, New Zealand.

### Oral Presentations:

"Moving Methyl Groups and Watching Them with NMR" Presented to the Department of Biochemistry, University of Sydney, Australia, July 2004.

"An NMR Based Assay For BHMT" Presented to the University of Otago, Dunedin, New Zealand, November 2003.

## Table of Abbreviations and Symbols

Whereever possible S.I. units and abbreviations have been used

Abbreviation	Definition
BHMT	Betaine Homocysteine Methyltransferase (E.C. 2.1.15)
<i>BHMT</i>	Betaine Homocysteine Methyltransferase gene
bp	Base Pairs
CBH	<i>S</i> -( $\gamma$ -carboxybutyl)-L-homocysteine
CBS	Cystathionine $\beta$ -Synthase (E.C. 4.2.1.22)
CVD	Cardiovascular Disease
CV	Coefficient of Variation
Da	Daltons (grams per mole), also kilo, and mega Daltons (1000, 1000000 Da, respectively)
DMG	<i>N,N</i> -Dimethyl Glycine
DMGDH	<i>N,N</i> -Dimethyl Glycine Dehydrogenase (E.C. 1.5.99.2)
DMS <sub>SeP</sub>	Dimethylseloniopropionate
DMSP	Dimethylsulfoniopropionate
DMS <sub>SeB</sub>	Dimethyl Seloniobetaine
DMT	Dimethyl Thetin
DNA	Deoxyribose Nucleic Acid
DPFGSE	Double Pulsed Field Gradient Spin Echo
DVT	Deep Vein Thrombosis
d1	Delay between NMR sequence iterations, measured in seconds
E.C.	Enzyme Commission number
<i>E. coli</i>	<i>Escherichia coli</i> bacteria
ESI-MS	Electrospray Ionisation Mass Spectrometry
FT-NMR	Fourier Transform Nuclear Magnetic Resonance
GCMS	Gas Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IF	Intrinsic Fluorescence
$K_m$	Michaelis Menten constant
MS	Methionine Synthase (E.C. 2.1.1.13)
mM	Milli moles per litre
mRNA	Messenger Ribose Nucleic Acid
MTAA	methylsulfinylmethanoate
mTHF	Methylene Tetrahydrofolate
m/z	Mass to charge ratio
NMR	Nuclear Magnetic Resonance
PCR	Polymerase Chain Reaction
ppm	Parts per million
rpm	Revolutions per minute
SAM	<i>S</i> -Adenosyl Methionine
SDS PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
THF	Tetrahydrofolate
TIA	Transient Ischemic Attack
TMA	Trimethylamine
TMAuria	Trimethylaminuria
TMAO	Trimethylamine <i>N</i> -Oxide
TRIS	<i>tris</i> -(hydroxymethyl)aminomethane
T1	Longitudinal relaxation time
T2	Transverse relaxation time
$V_0$	Void volume from size exclusion column
$V_e$	Elution volume from size exclusion column
$V_{max}$	Maximum rate from an enzyme catalysed reaction
WATR	Water Attenuation by Transverse Relaxation
WHO	World Health Organisation

## Chapter 1

# Introduction

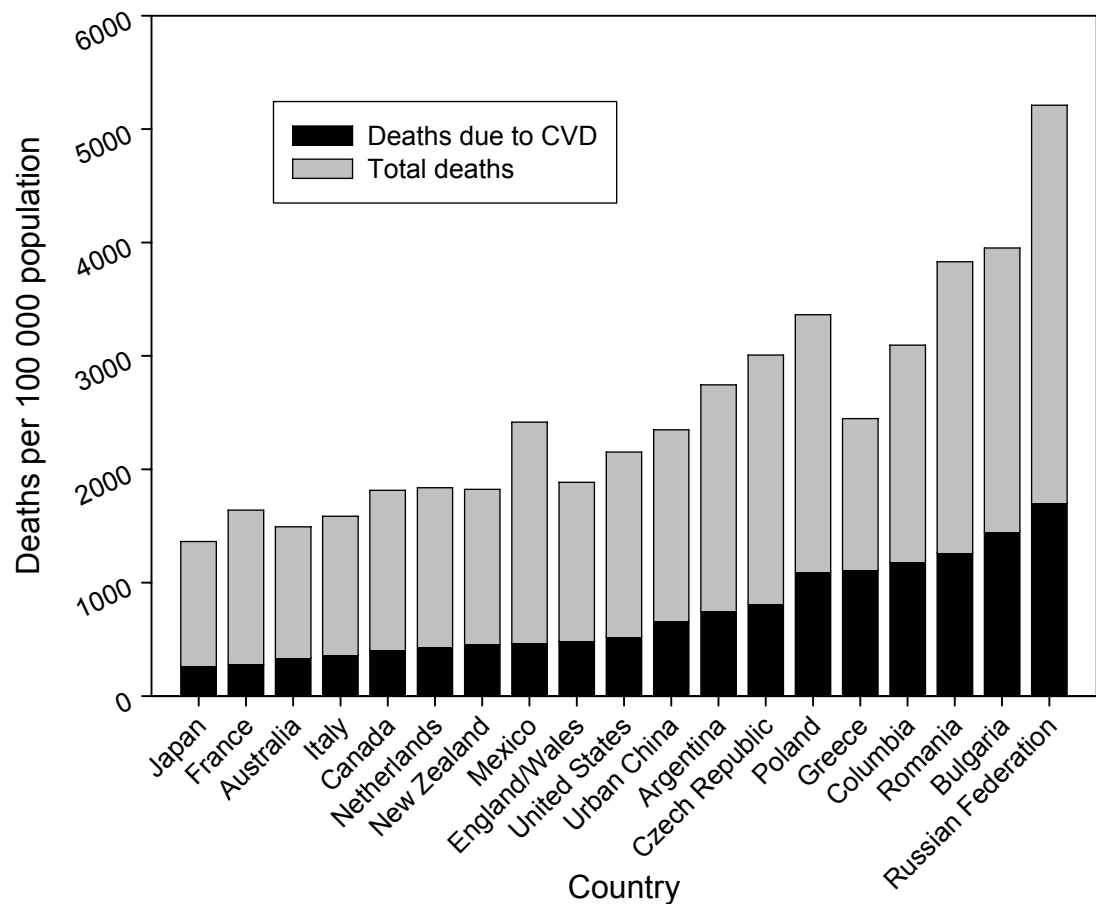
## CHAPTER 1: Introduction

### 1.1 Cardiovascular Disease

#### 1.1.1 The CVD Epidemic

Cardiovascular disease (CVD) is one of the major causes of death in the developed and developing world. The World Health Organisation (WHO) classifies CVD as “diseases which affect the proper functioning of the heart and blood vessels”, a description which incorporates myocardial infarction, cerebrovascular diseases (strokes), transient ischaemic attacks (TIA), deep vein thrombosis (DVT) and other peripheral vascular diseases.<sup>1</sup> According to the WHO, CVD kills some 17 million people every year, much of which can be attributed to poor lifestyle choices, such as smoking, diet and lack of physical activity.<sup>1</sup>

The New Zealand Heart Foundation estimates that 11% of the non-fatal disease burden in New Zealand is due to CVD, with 27,445 hospital admissions a year (1999 data). CVD is also the leading killer in New Zealand, responsible for 40% of all deaths. These statistics are comparable over the developed world, although the toll for developing nations is much higher. Worldwide, CVD is the single highest cause of mortality in women annually, with 8.5 million deaths (one third of all female fatalities). In developing countries, this figure accounts for over 50% of all female deaths. Developing regions are unfortunately not alone; 42% of all mortality in the European Union is due to CVD.<sup>1</sup> CVD is not just a disease of the old; 36% of premature deaths in men and 27% of premature deaths in women in the United Kingdom are due to CVD [Figure 1.1].



**Figure 1.1** Total death rate and CVD-death rate for selected countries (1997-2001).

### 1.1.2 Causes of CVD

There are many risk factors for CVD, including some metabolites of which the public are well informed. The most ill-famed of these is cholesterol, which has featured prominently in the literature since the 1950's.<sup>2</sup>

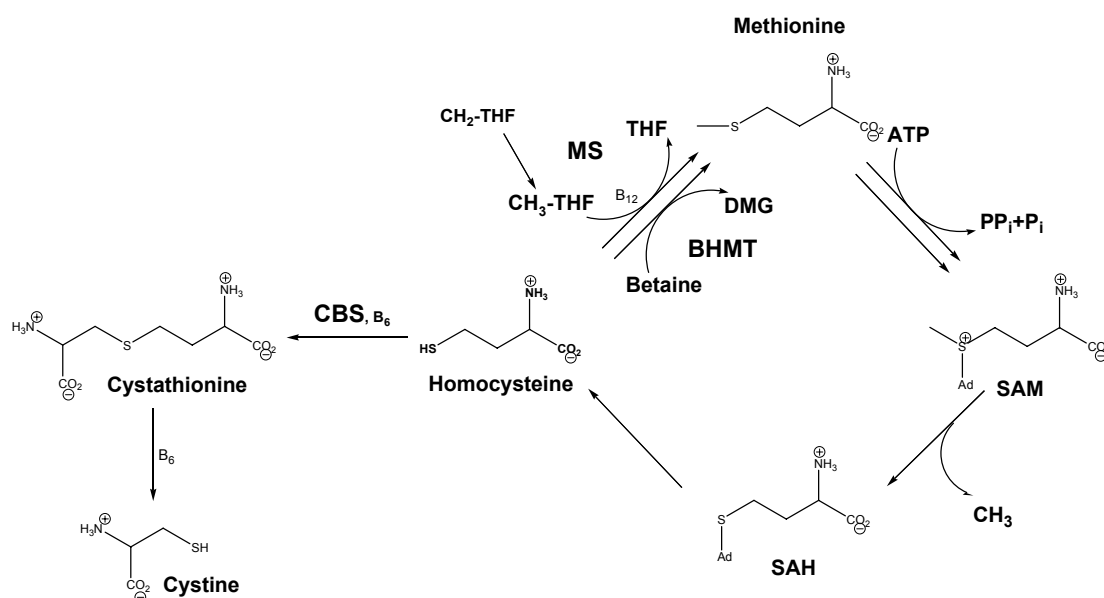
Smoking, high blood pressure, obesity, sex, age, race and genetics are all important factors involved in the incidence of CVD amongst many others.<sup>1</sup> It was identified during the 1960's that homocysteine levels are also an important predictor of CVD.<sup>3</sup> The link between homocysteine levels and CVD is now well established, although poorly understood. The link

may be causal, with a modest 3  $\mu\text{M}$  decrease in plasma homocysteine associated with a 16% decrease in TIA's, 25% decrease in DVT and 24% decrease in strokes.<sup>4</sup>

## 1.2 Homocysteine and Glycine Betaine

### 1.2.1 Homocysteine Biochemistry

Homocysteine is a non-protein amino acid, created by the removal of a methyl group from methionine, an essential amino acid for human beings [Figure 1.2]. Methionine is converted to *S*-adenosyl methionine (SAM), which is used by as many as 200 different enzymes as a methyl-donor. The product of this demethylation is *S*-adenosyl homocysteine, which can be hydrolysed into free homocysteine. Homocysteine can then be remethylated into methionine by two pathways; the methionine synthase pathway (E.C. 2.1.1.13, MS), which uses *N*<sup>5</sup>-methylene tetrahydrofolate as a methyl donor, and betaine-homocysteine methyltransferase (E.C. 2.1.1.5, BHMT), using glycine betaine (or its analogues) as methyl donors [Figure 1.2].



**Figure 1.2** Homocysteine metabolism (abridged). Homocysteine is remethylated by methionine synthase (MS) using tetrahydrofolate (THF), or by betaine homocysteine methyltransferase (BHMT) using glycine betaine and forming dimethylglycine (DMG). This is recycled via adenosine triphosphate (ATP) to *S*-adenosylmethionine (SAM) and then to *S*-adenosylhomocysteine (SAH). Homocysteine can also be broken down by cystathionine  $\beta$ -synthase (CBS) to ultimately form cysteine.

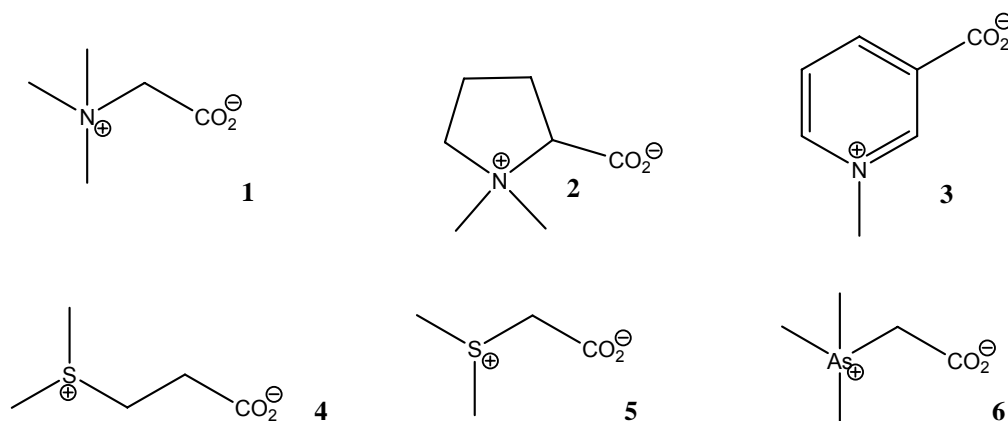
Homocysteine is also removed by the deconstruction of the backbone common to methionine and homocysteine (via cystathionine) ultimately to cysteine via the enzyme cystathionine  $\beta$ -synthase (CBS, E.C. 4.2.1.22) [Figure 1.2].<sup>5</sup>

Recent studies have discounted the influence of homocysteine on CVD by treating CVD patients with folate, and vitamins  $\text{B}_{12}$  and  $\text{B}_6$ .<sup>6,7</sup> All of these studies found that the folate and B vitamin therapy lowered total plasma homocysteine, but did not result in a lowered risk of further atherosclerotic events (with the exception of strokes, which were decreased). The conclusion, that homocysteine cannot be a causative factor in CVD, is premature, since they have ignored the BHMT pathway.<sup>8</sup> Furthermore, the results are difficult to reconcile with overwhelming data suggesting that homocysteine is implicated in CVD.<sup>4,9-14</sup>

## 1.2.2 Betaines

The term betaine refers to a class of compounds which are all zwitterionic (contain both a permanent positive and negative charge) at physiological pH. Glycine betaine was the first betaine to be discovered, originally from sugar beet, *Beta vulgaris*, hence the later naming of the class of compounds. Initially, glycine betaine was the only known compound that contains an *N,N,N*-methylated amino acid.

Since their original discovery, a number of other betaines have been isolated from nature, including many of the amino acids that have undergone similar methylation on the amine (or related heteroatom) moiety. Some of these compounds are shown in **Figure 1.3**.



**Figure 1.3** Some betaines used during the work for thesis. Glycine betaine (1), proline betaine (stachydrine, 2), trigonelline (3), dimethylsulfoniopropionate (DMSP, 4), dimethyl thetin (DMT, 5), and arsenobetaine (6).

Betaines have been implicated in several functions of normal cellular activity. These functions include regulating cell volumes and ionic strength, urea and solute protection of proteins (especially in the kidney) and methylation reactions (by acting as methyl donors). Salt tolerant organisms accumulate glycine betaine and other related betaines.<sup>15-17</sup> This accumulation restores cell volume, and balances osmotic pressure.<sup>18</sup>

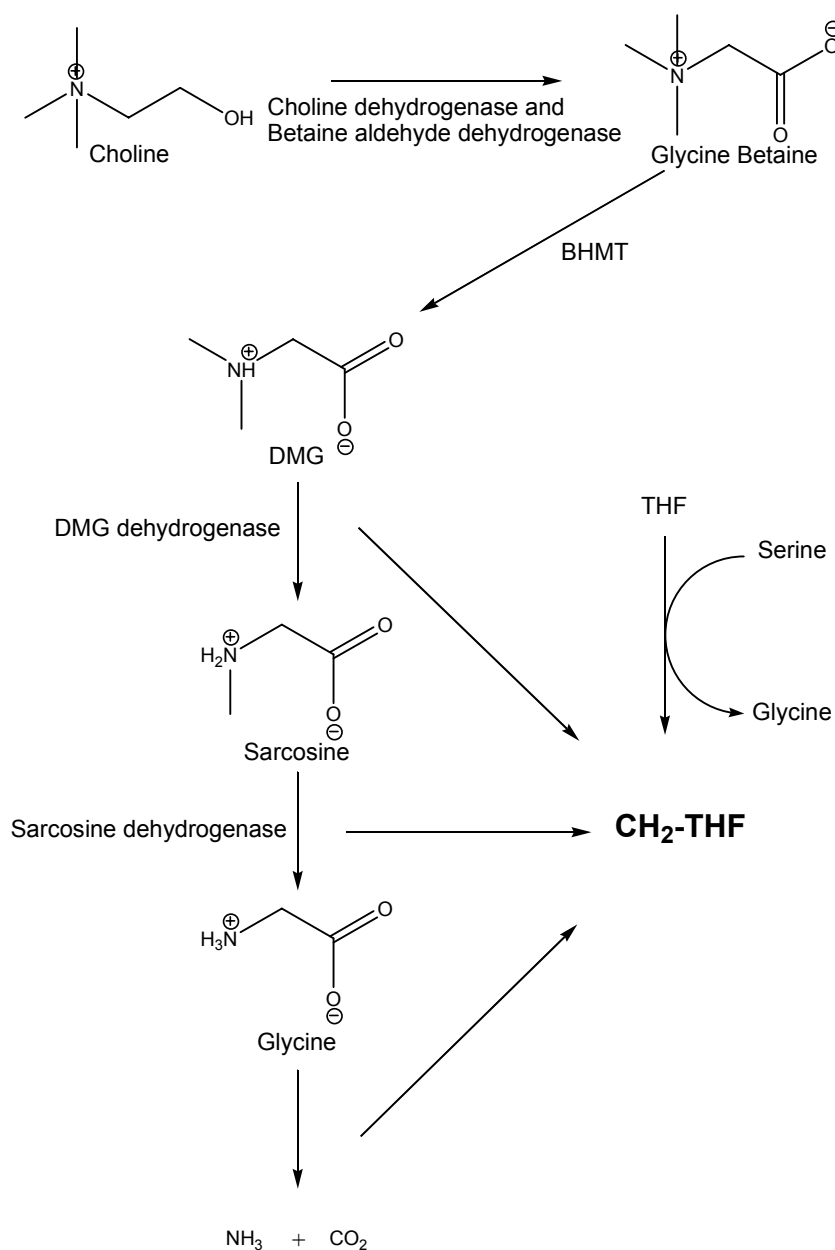


Betaines are utilised to protect against the macromolecular destabilising effect of urea on proteins.<sup>19,20</sup> There are several proposed mechanisms for this protection, including the 'Preferential Exclusion Model', in which macromolecules are protected by having a preferential interaction between betaine and the protein, over the urea and protein.<sup>21</sup> In this model, the protection comes from the positive and negative charges distributed across the betaine molecule.<sup>22</sup>

It is the methyl-donor properties of betaines, and their implications for homocysteine metabolism which will be the focus of this thesis.

### 1.2.3 Diet and Homocysteine

As Selhub points out, nutrition can play an integral part in homocysteine metabolism.<sup>23</sup> **Figure 1.2** illustrates the requirements for several substrates, vitamins and cofactors. Methionine synthase requires folate and vitamin B<sub>12</sub> as cofactors. Both steps in the destruction of the methionine/homocysteine backbone (from homocysteine to cystathionine, then to cystine) require vitamin B<sub>6</sub>. BHMT requires glycine betaine (which can be ingested or synthesized from choline) and zinc to facilitate the reaction between homocysteine and glycine betaine. Clearly, deficiencies in these vitamins and mineral will result in impaired homocysteine metabolism. Glycine betaine is acquired through the diet, and can also be synthesized from choline (also obtained endogenously) [**Figure 1.4**].<sup>24</sup> It has been recently claimed that humans cannot convert choline to the betaine,<sup>25,26</sup> and that humans are therefore dependent on dietary betaine,<sup>26</sup> but this is probably based on a misinterpretation of earlier work<sup>27</sup> that showed that humans had relatively low activity of choline dehydrogenase in liver compared with kidney activity, in contrast to the rat pattern. Dietary choline is undoubtedly an important source of glycine betaine and hence methyl groups in humans.



**Figure 1.4** Oxidation of choline to produce methylene-tetrahydrofolate (THF). Choline is oxidised in two steps to glycine betaine (via betaine aldehyde). This is then demethylated by betaine homocysteine methyltransferase (BHMT) to dimethylglycine (DMG). DMG can then demethylate to form sarcosine and one methylene tetrahydrofolate (CH<sub>2</sub>-THF). Sarcosine can then be demethylated to glycine and one CH<sub>2</sub>-THF. Finally, glycine can be metabolised into ammonia and carbon dioxide forming another CH<sub>2</sub>-THF.

### 1.2.4 Betaine Metabolism

The role of glycine betaine in homocysteine metabolism is not over once betaine has been demethylated by BHMT. Dimethylglycine (DMG) can be transported from the cytosol to the mitochondrion to be oxidatively demethylated to form sarcosine, by the enzyme dimethylglycine dehydrogenase (DMGDH, E.C. 1.5.99.2), to form methylene-tetrahydrofolate.<sup>28</sup> This pathway continues to oxidatively demethylate sarcosine to give another methylene-tetrahydrofolate, using sarcosine dehydrogenase (E.C. 1.5.99.1), resulting in glycine [Figure 1.4].<sup>28</sup>

Glycine can be further cleaved by the glycine cleavage system to form  $\text{CO}_2$ ,  $\text{NH}_3$  and a final methylene-tetrahydrofolate. In total, one choline molecule gives (via glycine betaine) a total of four methyl groups, and can potentially remethylate a total of four homocysteine molecules to methionine (folate is also used in the synthesis of purines, serine and many other biomolecules) [Figure 1.4].

The relative flux through each of these pathways is still a topic for debate. Finkelstein and Mudd provided the first evidence of this, showing that under non-physiological conditions and using liver extracts, MS, BHMT and CBS contribute 27%, 27% and 46% of homocysteine consumption respectively.<sup>29</sup>

Despite the evident importance of BHMT it has been largely ignored in the literature, (by medical professionals in particular). This is clearly evidenced by Selhub's review of 1999 "*...remethylation pathway to methionine, which requires folate and vitamin B12 (or betaine in an alternative reaction)...*".<sup>23</sup> The review largely ignores any potential BHMT influences on homocysteinaemia.

### 1.2.5 Homocysteine Disorders

The reference interval for plasma homocysteine concentration is 5-15  $\mu\text{M}$  ( $10.08 \pm 5.3 \mu\text{M}$ ).<sup>30,31</sup> Folate, vitamin B<sub>12</sub> deficiencies or mutations in methionine synthase (677C→T, for example) can result in plasma homocysteine concentrations of 20-50  $\mu\text{M}$ . Inborn errors in the CBS pathway (homozygous cystathionine  $\beta$ -synthase mutations) have been shown to increase plasma homocysteine concentrations, causing homocysteinaemia, giving plasma concentrations for homocysteine up to 400  $\mu\text{M}$ .<sup>23</sup> There is no known genetic defect in the BHMT gene leading to increased plasma homocysteine. A polymorphism (742G→A; R239Q) has been located and identified within patients attending the Mayo Clinic (Rochester, Minnesota), but this was not correlated with increased homocysteine (but was speculated to be linked to CVD).<sup>32</sup> This lack of BHMT mutations causing homocysteinaemia / homocysteinuria may be responsible for the lack of interest in BHMT.

Plasma homocysteine is the result of a cellular transportation mechanism, which removes homocysteine from the cytoplasm and maintains low cellular levels.<sup>33</sup> High plasma homocysteine concentrations are associated with older age, high creatinine concentrations, smoking, coffee consumption, high alcohol consumption, nitrous oxide and L-dopa treatments. Plasma homocysteine is higher in men than women.<sup>23</sup>

Patients with renal disease often have elevated homocysteine, up to four times normal concentrations.<sup>34</sup> The mechanism leading to this elevation is not clear, however, it may be a consequence of high excretion of glycine betaine and other cofactors associated with homocysteine metabolism.<sup>11,35</sup> Cardiovascular disease is also elevated in these patients, although homocysteine has yet to be established as the causative agent.

The sequence of events which leads homocysteine to cause CVD is not clear either. Homocysteine is rapidly auto-oxidised in plasma, forming dimeric homocystine (homocysteine disulfide), homocysteine thiolactone, and mixed disulfides with various other thiols, including

protein cysteine residues. Reactive oxygen species can be formed during this oxidation, including superoxide, peroxy species and hydroxyl radicals.<sup>34</sup>

Evidence is mounting indicating that homocysteine-induced damage to endothelial cells within the walls of the circulatory system (mediated by hydrogen peroxide) exposes smooth muscle cells and matrix, proliferating and activating platelets and leukocytes.<sup>34</sup>

Endothelial cells are not completely defenceless against homocysteine. Stamler *et al*/ showed that endothelial cells can produce nitric oxide, which combines with homocysteine in the presence of oxygen, detoxifying homocysteine to *S*-nitroso-homocysteine.<sup>36</sup> This prevents peroxide production from homocysteine oxidation, relieving the oxidative stresses on the endothelial cells. *S*-Nitroso-homocysteine also acts as a platelet inhibitor and vasodilator.<sup>34</sup> However, the endothelial cells cannot withstand this treatment indefinitely, damage to the endothelium eventually affects the production of nitric oxide, and oxidative damage begins in earnest.

Homocysteine also promotes the production of oxy-cholesterols, a highly atherogenic class of compounds, oxidises low-density lipoproteins, and increases lipid peroxidation. This would potentially explain the multiplicative effect of homocysteine on other CVD risk factors (like cholesterol and lipid levels).<sup>11</sup>

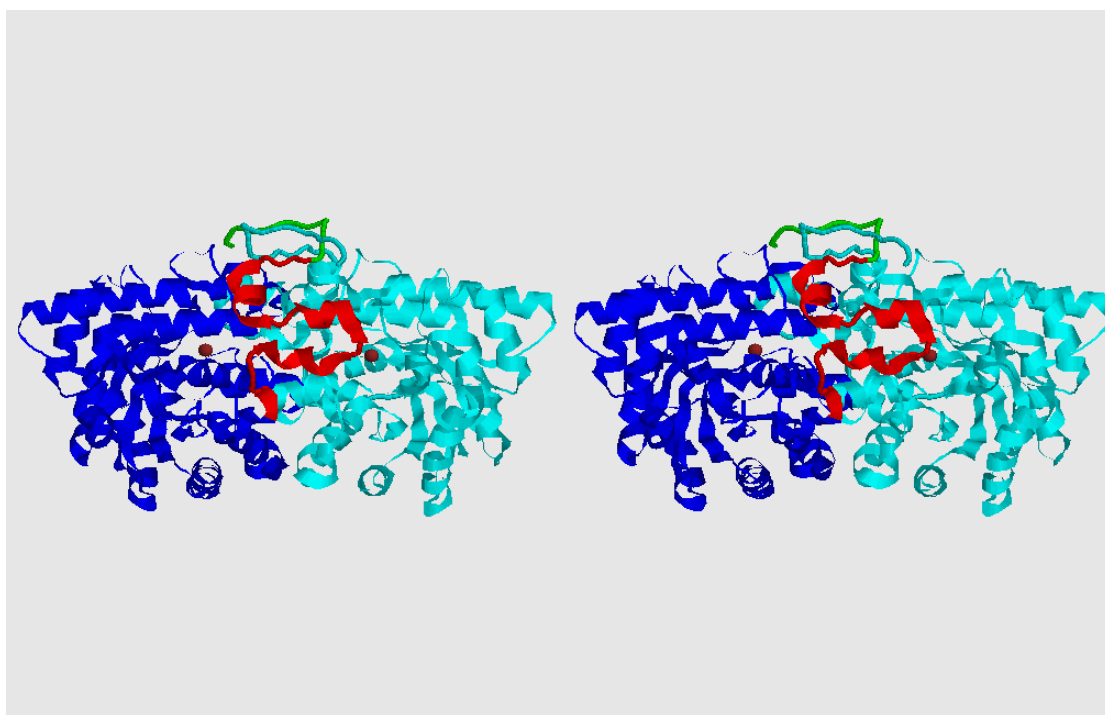
### 1.3 Betaine Homocysteine Methyltransferase

#### 1.3.1 BHMT

BHMT has a moderately large subunit of 44.5 kDa (calculated from the open reading frame of the human BHMT gene – coding for 406 amino acids).<sup>37</sup> There has been a vigorous debate regarding the quaternary structure of BHMT. Early reports suggested that BHMT was a hexamer (following size exclusion chromatography), while the tetramer was identified, but

largely discounted.<sup>38-40</sup> More recent studies would suggest that BHMT is active as a tetramer, not as a hexamer.<sup>41,42</sup>

Early structures released by Evans *et al*/ showed a 'dimer of dimers'; a tetrahedral arrangement of four subunits [Figure 1.5]. However, they could not describe any mechanism for this arrangement – there appeared to be little interaction between the subunits to allow this organisation.



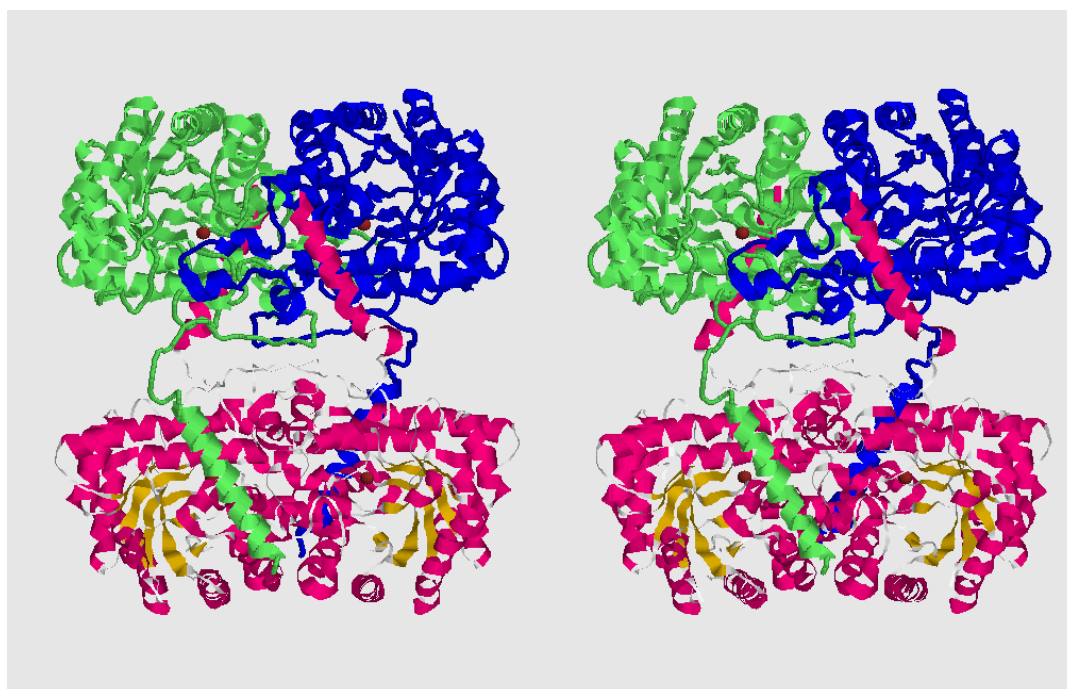
**Figure 1.5** Evans *et al*/ crystal structure for recombinant BHMT (stereo-view). The 'dimerisation arm' and 'hook region' of subunit A have been coloured red and green, respectively.

Their structure did, however, reveal the organization between subunits, leading to dimerisation. They describe this region as the 'hook region', shown at the top of Figure 1.5. The hook-shaped loop at <sup>362</sup>PYNP<sup>365</sup> wraps about the corresponding hook region of the second subunit, forming a tight link and enabling dimerisation.

There is a second region named the 'dimerisation arm' that extends from the TIM barrel motif from residue 318 (visible in dark blue in Figure 1.5 above) and contributes inter-chain interactions that assist in dimer formation. The structure has a number of disordered

regions, from residues 2-10, 38-52, 76-99, 325-332 and 372-407; the active site, however, can be seen. Molecular modelling of the region 372-407 at the C-terminal end of the polypeptide predicted an  $\alpha$ -helix, but this could not be resolved in the crystal structure.<sup>41</sup>

Gonzalez *et al*/ published the complete crystal structure in early 2004 [Figure 1.6]. Their structure contains the remainder of the structure, revealing further information about the previously disordered regions.<sup>42</sup> Their structure shows that the C-terminal region (residues 372-407) does form an  $\alpha$ -helix, and also form interactions between dimers, allowing tetramerisation.



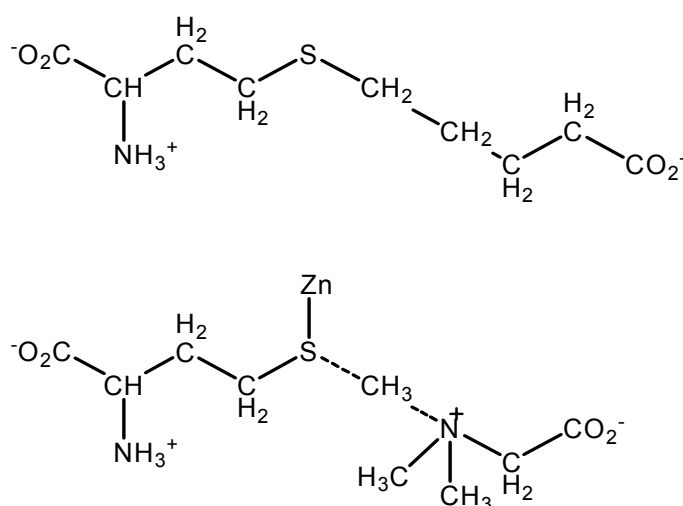
**Figure 1.6** Gonzalez *et al*/ structure of Rat Liver BHMT (stereo-view).<sup>42</sup>  $\text{Zn}^{2+}$  has been included as a brown 'spacefilled' point. Subunits A and B are coloured by structure to show  $\alpha$ -helix (pink) and  $\beta$ -sheets (yellow).

BHMT is a zinc metalloenzyme,<sup>43</sup> containing a highly conserved zinc binding motif of three cysteine residues, holding the zinc ions in an approximate tetrahedron.<sup>41</sup> In rat, mouse, pig and human BHMT the zinc motif is always <sup>214</sup>GVNCH<sup>218</sup> and <sup>293</sup>VRYIGGCCGFEPYHI<sup>317</sup> (amino acid numbers for rat BHMT).<sup>44</sup>

## 1.3.2 BHMT Mechanism

In 1983, Awad *et al* found that a homocysteine analogue, *S*-( $\delta$ -carboxybutyl)-L-homocysteine (CBH) was a potent inhibitor ( $K_i \sim 3.25 \mu\text{M}$ ) of their recently isolated BHMT extract.<sup>45</sup> They inferred that CBH was mimicking the transition state of the reaction, further implying that there was a direct transfer of a methyl group from glycine betaine to homocysteine in an  $\text{S}_\text{N}2$ -type reaction [Figure 1.7].

This information did not, however, give any information as to the enzyme conformational changes that may be occurring in the tertiary/quaternary structure of the protein which enable the methyl-transfer.



**Figure 1.7** *S*-( $\gamma$ -carboxybutyl)-L-homocysteine (CBH - top) and the proposed transition state for BHMT (bottom).

Further evidence came with the application of intrinsic tryptophan fluorescence to the mechanism of BHMT.<sup>46</sup> Tryptophan is highly fluorescent, and its fluorescent properties are sensitive to changes in chemical environment. Proteins that contain tryptophan will often undergo changes in fluorescence as they alter their conformational state.<sup>47</sup> Site directed



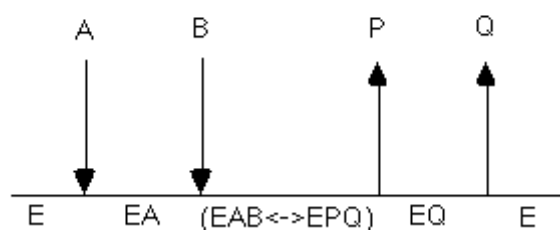
mutagenesis can identify which tryptophan residues are responsible for the changes in fluorescence and, in turn, give information about the catalytic activity of the enzyme.

Castro *et al*/identified a change in fluorescence intensity and emission wavelength (a 4 nm shift) when methionine and homocysteine were incubated with recombinant human BHMT. No change was observed with BHMT in the presence of betaine or DMG. When BHMT was incubated with homocysteine and betaine (or DMG) a second shift in emission wavelength (an 8 nm shift) and intensity was seen. An almost identical shift was seen with the incubation of BHMT with CBH. It was also revealed that betaine binding was only possible after homocysteine had bound. Furthermore, fluorescence changes due to Trp 342 (part of the dimerisation arm) showed that betaine binding was assisted by the second monomer.

This experiment established that the mechanism for recombinant human BHMT was an ordered 'bi-bi' mechanism – homocysteine must bind before betaine can access its binding site and DMG must leave before methionine.

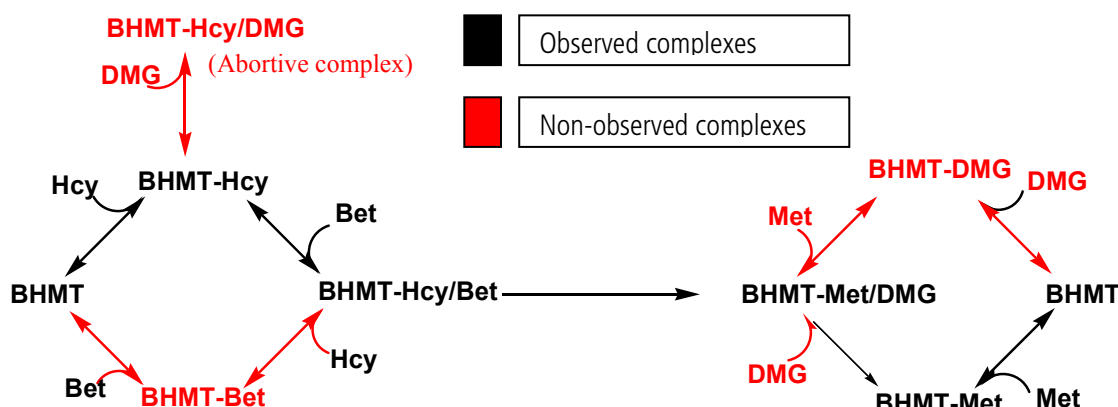
Systematic site directed mutagenesis of the seven tryptophan residues in BHMT identified Trp44 as being part of the active site, forming a hydrogen bond to the CBH carboxylate. Due to changes in fluorescence emission wavelength for the mutated proteins, it is clear that tryptophans 169, 342, 352 and 373 undergo changes in environment during catalysis/substrate binding. Tryptophans 331, 342 and 352 are all located on the dimerisation arm of the partner subunit – giving rise to interactions between subunits and catalytic activity.

The intrinsic fluorescence (IF) data also showed that BHMT has an 'ordered bi-bi' mechanism. Enzyme mechanisms involving the binding of more than one substrate can be complicated. In ordered bi-bi mechanisms each substrate binds in a defined order, the reaction occurs, and then the products leave the enzyme in a second, defined order [Figure 1.8]. Most often, the binding of one substrate creates the binding site for the second substrate.



**Figure 1.8** Ordered bi-bi mechanism schematic. Enzyme (E) initially binds substrate A, forming a complex (EA). This then binds substrate B forming an EAB complex, which reacts to form the EPQ complex. Product P leaves the enzymes, leaving complex EQ, followed by Q undocking from the enzyme, leaving free enzyme E.

The IF data presented by Castro *et al*/showed that BHMT does not bind glycine betaine until after homocysteine has bound. Furthermore, BHMT does not release methionine until after DMG has departed the binding site. This also explains the strong inhibition by DMG, which would be expected to bind in the glycine betaine site, but would not react, giving an abortive complex. Overall, the mechanism for BHMT activity is shown below in **Figure 1.9**.



**Figure 1.9** BHMT mechanism as determined by IF spectroscopy. BHMT binds homocysteine, and then glycine betaine (bet), forming a complex which reacts to give BHMT-Met/DMG complex. DMG then leaves the complex giving BHMT-Met. Methionine then leaves BHMT leaving the free enzyme.

### 1.3.3 BHMT Distribution

BHMT appears to be highly conserved in mammals, with the human BHMT gene sharing 88% identity to the porcine gene, and 94% amino acid identity.<sup>48</sup> BHMT has been purified to homogeneity from horse and rat.<sup>38</sup> These preparations lead to the conclusion that some 0.6-1.6% of all the soluble protein in the liver is BHMT.<sup>37</sup> There have been claims that BHMT activity can be found in other taxa, including fungi (*Aspergillus nidulans*),<sup>49</sup> and bacteria (*Rhizobium meliloti* and *Pseudomonas denitrificans*).<sup>49-51</sup>

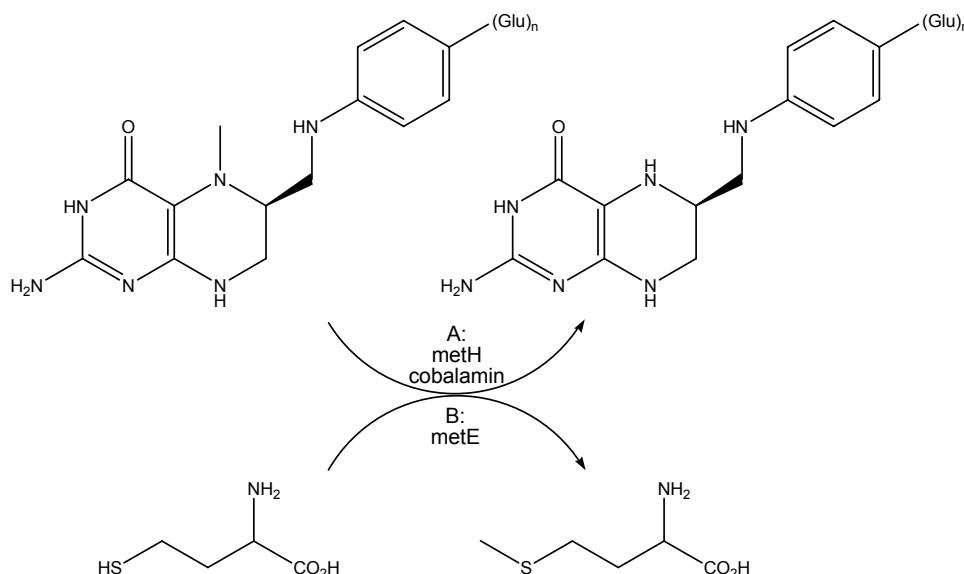
BHMT has been located within the liver and kidneys of most mammals, but its distribution within other tissues seems to be species specific.<sup>51-53</sup> In rats, there appear to be very low levels of activity in the kidney.<sup>51</sup> BHMT has been found within the eye lens of rhesus monkey,<sup>54</sup> in pancreas from sheep,<sup>55</sup> and in brain tissue from human.<sup>56</sup> No activity has been found in rhesus monkey or pig brain. Sunden *et al* studied the mRNA for BHMT within different human tissues by northern analysis, and found that BHMT was expressed in the liver and kidney, but not the lung, heart, brain, skeletal muscle or pancreas.<sup>53</sup> By western analysis, BHMT has been located in pig kidney cortex and liver, but again, not in the lung, heart, spleen or brain.<sup>53</sup>

The enzymes introduced earlier, involved in choline metabolism and oxidation (DMGDH and sarcosine dehydrogenase [Figure 1.4]) are also localised mainly in the liver and kidney. Methionine synthase and CBS are all expressed ubiquitously; the only homocysteine methylation enzyme to show tissue specificity is BHMT.

Mammalian BHMT was shown to share similarities to *N*-terminal sequences of bacterial and eukaryotic methionine synthases, as well as having a common zinc binding motif.<sup>37</sup> From this sequence, BHMT was allocated to the pfam 02574 family of thiol/selenol methyltransferases. These proteins all facilitate the movement of a methyl group to or from a selenol or thiol group.

## 1.3.4 Methionine Synthase

Bacterial methionine synthase is an example of a one carbon transferase, which is used to convert homocysteine to methionine in *E. coli*. It is also a member of the pfam 02574 family of S/Se-methyltransferases. There are two enzymes which utilise this general reaction, however, the similarity is superficial, and only one of these belongs to the same pfam as BHMT. One enzyme (cobalamin-dependent methionine synthase, methH) uses cobalamin as a cofactor to facilitate the reaction, while the other (cobalamin-independent methionine synthase, metE) functions without the assistance of cobalamin [Figure 1.10]. Both use *N*<sub>5</sub>-methyl-tetrahydrofolate (Me-THF) as the methyl group donor, and produce methionine and tetrahydrofolate (THF) products. The methH protein has homologues in animals (including mammals) and eubacteria, but not in plants or archaeobacteria. MetE has homologues in plants, archaeobacteria, yeast, insects and eubacteria, but not in mammals.<sup>57</sup>



**Figure 1.10** Methionine synthase reactions. A is the cobalamin dependent pathway, B is the cobalamin independent pathway.

In the metE pathway, homocysteine directly attacks the *N*<sub>5</sub>-methyl group; the methH enzymes involve the transfer of methyl groups to cobalamin, forming methyl-cobalamin. These

are then used to methylate homocysteine, forming methionine and cob(I)alamin, followed by transfer of a methyl group from Me-THF to cob(I)alamin to regenerate the methyl-cobalamin and forming THF.

With regard to metE, tertiary amines are not good methyl-donors. The  $pK_a$  of the anionic THF leaving group is  $>30$ . However, if protonation occurs before the methyl transfer, then the resulting secondary amine has a  $pK_a$  of about 5.<sup>58</sup> Without the intervention of an enzyme, only one in every 100 molecules of Me-THF will be in a state ready for reaction. What is more, the  $pK_a$  of the thiol group on homocysteine is about 10 and is, therefore, not considered to be nucleophilic.<sup>59</sup> Only about one in every 1000 molecules of homocysteine would be in a reactive thiolate form at pH~7.

In the event that a protonated Me-THF would react with a thiolate in an enzyme-free solution, proton transfer would be a more kinetically favourable process. MetH avoids this problem by introducing a cobalamin intermediary to facilitate the methyl transfer. However, in metE (and similarly in BHMT) there is no such intermediary, and thus the  $pK_a$  of the Me-THF must be increased and the  $pK_a$  of homocysteine must be lowered via some alternative mechanism.

MetH and metE both contain zinc, although the binding site varies between these two enzymes. MetE uses a His-Zn-Cys with a second downstream Cys to hold zinc, while metH uses a Cys-Cys functionality with an upstream Cys, a similar motif to BHMT's zinc binding site. Zinc plays an important role in all of these homocysteine remethylations, acting as a Lewis acid catalyst. By binding to sulfur, zinc can decrease the  $pK_a$  of homocysteine until the thiolate form predominates at neutral pH.

BHMT also contains the same binding motif, and binds homocysteine before any reaction takes place.<sup>44</sup> It is not unreasonable to infer that similar mechanisms must be in play to allow the reaction between homocysteine and betaine. Both THF and glycine betaine are

nitrogenous alkylating agents. More importantly, there is evidence that in one eukaryote (the fungus *Aspergillus nidulans*) the same enzyme is responsible for both MS activity and BHMT activity.<sup>49</sup> The BHMT activity in *Aspergillus* requires significant verification, as more recent research suggests that there may not be any activity at all.<sup>60</sup>

### 1.3.5 BHMT Genetics

The gene coding for BHMT in humans, *BHMT*, was identified by Garrow and Park in 1999. It was found to contain 8 exons and 7 introns.<sup>61</sup> The BHMT protein has been expressed and purified from cDNA in *Escherichia coli* (*E. coli*).<sup>37</sup> It was found that there is a polymorphism, which appears to have no biological effect on BHMT biochemistry.<sup>62</sup> There was an identifiable TATA protein binding site 25 base pairs 5' to the transcriptional start site. Furthermore, there are 6 putative SP1 sites clustered about the TATA box, and further 5' there are apparent HNF-1, HNF-3 and CAAT enhancer-binding protein sites. These sites have all been identified as liver specific (or liver enriched) transcription factors.<sup>61</sup>

Interestingly, a *BHMT-2* gene has been identified in mice and humans.<sup>63</sup> mRNA corresponding to *BHMT-2* has been located and identified in the liver and kidneys in humans, and is also found at reduced concentrations in the brain, heart, and skeletal muscle. Mouse *BHMT-2* cDNA is 79% identical to *BHMT* cDNA and the theoretical protein that it corresponds to (BHMT-2) is 69% identical (amino acid sequence) to the BHMT protein.<sup>63</sup> The mouse *BHMT-2* cDNA is 87% identical to the human *BHMT-2* cDNA and the amino acid sequences that they code for are 82% identical.<sup>63</sup> Human BHMT-2 has a theoretical weight of 40.3 kDa, and shares 69% amino acid identity with human BHMT.<sup>63</sup>

### 1.3.6 BHMT Regulation

*BHMT* expression is highly regulated by hormones and also by feedback inhibition. *BHMT* is controlled via interactions with the BHMT protein, as well as by regulation of the expression of BHMT. Early studies of BHMT showed that activity in rat liver was elevated after protein loading.<sup>64-66</sup> Furthermore, methionine injections were able to stimulate increased activity of BHMT, while methionine synthase (MS) activities were suppressed, both by high protein intake and methionine injections. Finkelstein *et al*/ concluded that MS was used to regulate methionine during periods of reduced dietary intake of methionine, while BHMT was used to reduce homocysteine concentrations and facilitate choline catabolism.

In 1982, Finkelstein *et al*/ undertook a study to further explore the effects of dietary stress on BHMT activity.<sup>67</sup> By diminishing the intake of methionine, while also reducing choline concentrations, the group were hoping to observe steady activity in BHMT, and increased activity in MS. It was discovered that, contrary to previous experience, BHMT activity was increased as well as MS. Adding methionine to the diet suppressed the BHMT and MS levels to previous levels. It was inferred from these results that *BHMT* was activated by increases in homocysteine levels, as well as betaine concentrations. Thus, supplementing the diet with protein or methionine will produce higher homocysteine concentrations, and yield a greater activity of BHMT. Reduction in methionine in the diet can produce a lowering in plasma homocysteine, creating a lowered BHMT activity.

Finkelstein *et al*/ observed the interactions between methyl-donor (betaine) and BHMT in the rat.<sup>68</sup> It was found that betaine loading induced BHMT activity, implying that BHMT was regulated by tissue concentrations of glycine betaine. Finkelstein *et al*/ hypothesised that BHMT was used to catabolise excess betaine.

Research on rats can be complicated by their coprophagic behaviour, and so work during the 90's turned towards chickens.<sup>69</sup> Chickens have a number of favourable aspects,

lending themselves to studies of one-carbon metabolism. They utilise a BHMT enzyme in similar tissues to mammalian subjects at comparable concentrations with (what appears to have) similar enzymatic properties, do not practice coprophagy, and have diets which can be easily manipulated. Emmert *et al*/ confirmed the increase in BHMT activity post methionine loading, but also showed that a deficiency of methionine elevated BHMT by 300-500%. This could be enhanced by the addition of choline (a betaine precursor) into the diet.

It is clear that dietary betaine and methionine intake can influence *BHMT* expression; *BHMT* is also regulated by other factors, including developmental stage. BHMT has been found in the lens of rhesus monkey eyes acting as a crystallin (while retaining catalytic activity).<sup>54</sup> The inner nucleus of the eye contains cells which are preserved from embryonic tissue to adult, and have developmentally lost their nuclei; therefore no active production of protein can occur. BHMT makes up ~10% of the total protein in the central regions of the monkey lens; five times the concentration reported for pig liver.<sup>37</sup> *BHMT* must, therefore, be expressed during embryonic stages of development. The lens cells produced later in life do not contain high BHMT activities; the protein must undergo developmental regulation.

The lens of monkey eye does not contain any significant level of betaine. The lens is highly susceptible to osmotic stress, a contributing factor in some cataract development. Betaine is synthesised by the oxidation of choline in the mitochondrion, however, this organelle is lost in the cells of the inner lens. No betaine production is possible, and so any betaine used by the BHMT present must have diffused from circulating levels (complicated by the lack of blood vessels in the lens). In short, the presence of BHMT in the eye is unlikely to be due to catalytic requirements, unless high betaine is present during development.

In sheep, BHMT activities have been shown to vary with development, as do the activities of many other methyl transferases. BHMT activity in the liver is at its highest 40 days after birth (~250 pmol/min/mg protein), after which the activities drop to around half those



levels (~125 pmol/min/mg protein).<sup>55</sup> Strangely, sheep have high levels of BHMT activity in the pancreas, which also seems to undergo developmental changes, from relatively low activities during foetal stages (~100 pmol/min/mg protein) to levels 400% higher than the liver (~1000 pmol/min/mg protein).

There is also considerable evidence that *BHMT* regulation occurs through hormonal control. Finkelstein *et al*/noted a change in hepatic BHMT activity in lab rats after treatment with various hormones.<sup>66</sup> Hydrocortisone produced a 300% increase in activity, while thyroxine reduced activity to 35% of normal levels. They also noted that activities were not affected by gender of the rat, implying that sex-hormones do not affect the BHMT enzyme. Interestingly, stress has been observed to lower homocysteine in animals.<sup>70</sup> It is tempting to draw a link between hydrocortisone and this stress response. They conclude that there is an increased load on the cystathionine- $\beta$ -synthase pathway – increasing the cysteine production to decrease the oxidative load which is experienced in acute stress.

Most recently interest has turned to BHMT playing a role in controlling osmotic stress within the kidney. Betaine is an organic osmoprotectant, and is accumulated under hypertonicity in a number of different cells, particularly those of the kidney medulla. As the medulla concentrates urine it undergoes extremes of osmotic pressures.

The medulla uses several mechanisms to accumulate and retain betaine, it can increase transport of betaine by increasing expression of a  $\gamma$ -aminobutyrate/betaine transporter (BGT1).<sup>71</sup> The other major mechanism involves the oxidation of choline to betaine within medullary cells. In the event of hypernatraemia, choline dehydrogenase activity increases in the kidney.<sup>72</sup>

Delagado-Reyes and Garrow showed that BHMT is also down-regulated during these hyperosmotic periods in the guinea-pig by feeding them on salted water.<sup>73</sup> This treatment has two effects, increasing sodium concentration, and also dehydrating the animal (which responds

to the treatment by reducing its water consumption). However, the effect was clear – protein concentration and BHMT activity dropped in the liver and the kidney. The mechanism of this decrease was not investigated, and it may be due to decreased transcription or increased protein degradation.

The pioneering work of Finkelstein *et al* (1971) showed that the BHMT activity in rat liver is controlled by a number of hormones, with cortisol (increasing BHMT expression), thyroxine and insulin (both decreasing expression) being major candidates.<sup>66</sup> Thyroxine control of mammalian BHMT expression had earlier been suggested,<sup>74</sup> and has been subsequently demonstrated in chickens.<sup>75</sup> Consensus binding sites for steroid hormone receptors are associated with the *BHMT* gene.<sup>61</sup> Recently, the control of BHMT expression by insulin has been supported in rats,<sup>76,77</sup> and the effect of corticosteroids confirmed.<sup>77</sup>

### 1.4 Assays for BHMT

#### 1.4.1 BHMT Assays

BHMT is an important enzyme, due to its potential links with heart disease. Yet, despite its importance, relatively little attention has been drawn to it in the literature. BHMT activity has been observed using three main methods, each of which has had advantages, and disadvantages.

#### 1.4.2 Radiochemical Assays

By providing BHMT with a source of radiolabelled glycine betaine and L-homocysteine, BHMT activity can be monitored by production of radiolabelled methionine after a known period.<sup>39,78,79</sup> This method can be modified to give kinetic data, if the initial rate period is known, and the reaction can be terminated before the reaction reaches this point.

This technique suffers from one major problem. The radiolabelled substrates can be difficult to synthesise in the laboratory if they are not commercially available. In a study of substrate specificity and kinetics (on alternative substrates), the lack of radiolabelled substrates make this an unacceptable method to use. Also, the inability to conveniently produce activity-time graphs makes the determination of initial rate kinetics difficult.

#### 1.4.3 HPLC Methods

HPLC can also be used to analyse the metabolites from the BHMT reaction. Betaines are colourless and have no useful UV absorption, however they can be conjugated with fluorescent molecules or 'tags'. The tagged betaines are relatively easy to separate on standard anion-exchange HPLC columns.<sup>35,80-82</sup> Using similar conditions to those employed in

the radiolabelled methods for BHMT detection, the substrates can be incubated with the enzyme, and later analysed for methionine or betaine concentration.

This technique suffers from a similar problem to the radiolabelled assay. The initial rate data can be difficult to determine without continuous data (also giving rise to high uncertainties). There is also no guarantee that the fluorescent tags will work with all potential betaine analogues of interest, and these may need modification to enable quantification of analogues of glycine betaine. For these reasons, this assay is unsuitable for the analysis of BHMT substrate specificity.

### 1.4.4 *In Vivo* Bacterial Assay

In response to the need for a sensitive assay which could identify potential substrates for BHMT, Garrow *et al* developed an *in vivo* assay for BHMT, utilising bacterial growth in a minimal medium. *E. coli* J5-3 is a methionine auxotroph; it cannot synthesise a supply of its own. As a consequence, if J5-3 is provided with a plasmid encoding BHMT activity and a viable source of methyl donors, it will be capable of growth, and a measure of optical density will be a measure of the activity of the BHMT enzyme.<sup>83</sup>

One drawback to this assay is the dependency on bacteria: the betaine product of the BHMT reaction may have antibiotic properties – a property which some betaine analogues have, as noted by Chambers and Lever.<sup>84</sup> However, Garrow *et al* used the assay to show that the thetins are substrates, and that proline betaine could also be a substrate.

The microbiological assay is also incapable of giving kinetic results, a frustrating setback if a potential substrate is found, and cannot be completely characterised. Furthermore, it is likely that the sensitivity seen in this assay is a result of a non-linear response at low levels

of methionine production, making low levels of BHMT activity difficult to quantify. This does, however, make the microbiological assay good for detecting trace levels of activity.

### 1.4.5 Nuclear Magnetic Resonance (NMR) Assays

The naturally occurring betaines, broadly speaking, all contain methyl groups attached to a hetero-atom. This is the functionality which gives rise to their cationic centre, and partially contributes to their zwitterionic and kosmotrophic properties.

As a direct consequence of this moiety, these methyl groups all appear as singlets in proton NMR spectra, often well separated from resonances arising from other functionalities. Glycine betaine methyl signals appear as a strong singlet at 3.22 ppm, and the BHMT products DMG and methionine methyl signals appear as singlets at 2.88 ppm and 2.10 ppm, respectively. All three signals can be observed simultaneously so the progress of the BHMT reaction can be monitored by analysis of these three parameters (loss of betaine signal, and increase in DMG and methionine signals). What is more, potential analogues for the BHMT reaction will also contain methyl groups, and are likely to generate signals in different regions of the NMR spectrum from glycine betaine, DMG and methionine.

NMR spectroscopy can be used to quantify chemicals in complex mixtures so magnetic resonance spectroscopy lends itself as an ideal technique for the analysis of BHMT activity. It is capable of analysing multiple substrates and products, is rapid, non-destructive, can be automated, and it enables kinetic analysis of substrates and inhibitors.

### 1.5 Thesis Aims

Most of the aims in this thesis involved the application of  $^1\text{H}$ -NMR spectroscopy to biological samples. Specifically the aims were:

1. To develop a reproducible method for the purification of BHMT from liver samples [Chapter 2].
2. To develop a  $^1\text{H}$ -NMR spectroscopic assay for BHMT for the purposes of detection of BHMT, and measurement of kinetic constants for BHMT [Chapters 3 and 4].
3. To use  $^1\text{H}$ -NMR spectroscopy to investigate the substrate specificity of BHMT from rat liver [Chapter 5].
4. The observation of BHMT from alternative sources, including Agnathan liver samples, and to partially characterise the enzyme for comparison with rat BHMT [Chapter 6].
5. To use  $^1\text{H}$ -NMR spectroscopy to measure methylamines and related compounds in urine as an indirect method for the detection of BHMT activity *in vivo* [Chapter 7].

### References

- (1) Tunstall-Pedoe, H. *MONICA Monograph and Multimedia Sourcebook: World's largest study of heart disease, stroke, risk factors and population trends 1979-2002* Geneva, 2002.
- (2) Albrink, M. J. *Journal of the American Dietetic Association* **1965**, Diet and Cardiovascular Disease, *46*, 26-29.
- (3) McCully, K. S. *American Journal of Pathology* **1969**, Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis, *56*, 111-128.
- (4) Wald, D., S.; Law, M.; Morris Joan, K. *British Medical Journal* **2002**, Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis, *325*, 1202.
- (5) Finkelstein, J. D. *European Journal of Pediatrics* **1998**, The metabolism of homocysteine: pathways and regulation., *157*, S40-S44.
- (6) Lonn, E.; Yusuf, S.; Arnold, J.; Sheridan, P.; Pogue, J.; Micks, M.; McQueen, M. J.; Probstfield, J.; Fodor, G.; Held, C.; Genest, J., Jr. *New England Journal of Medicine* **2006**, Homocysteine lowering with folic acid and B vitamins in vascular disease, *354*, 1567-1577.
- (7) Bonaa, K. H.; Njolstad, I.; Ueland, P. M.; Schirmer, H.; Tverdal, A.; Steigen, T.; Wang, H.; Nordrehaug, J. E.; Arnesen, E.; Rasmussen, K. *New England Journal of Medicine* **2006**, Homocysteine lowering and cardiovascular events after acute myocardial infarction, *354*, 1578-1588.
- (8) Loscalzo, J. *New England Journal of Medicine* **2006**, Homocysteine trials - Clear outcomes for complex reasons, *354*, 1629-1632.
- (9) Olthof, M. R.; Verhoef, P. *Current Drug Metabolism* **2005**, Effects of betaine intake on plasma homocysteine concentrations and consequences for health, *6*, 15-22.
- (10) Lever, M.; George, P. M.; Dellow, W. J.; Scott, R. S.; Chambers, S. T. *Metabolism, Clinical and Experimental* **2005**, Homocysteine, glycine betaine, and N,N-dimethylglycine in patients attending a lipid clinic, *54*, 1-14.
- (11) Austen, S. K.; Coombes, J. S.; Fassett, R. G. *Nephrology* **2003**, Homocysteine and cardiovascular disease in renal disease, *8*, 285-295.
- (12) Perna, A. F.; Ingrosso, D.; Castaldo, P.; De Santo, N. G.; Galletti, P.; Zappia, V. *Mineral and Electrolyte Metabolism* **1999**, Homocysteine, a new crucial element in the pathogenesis of uremic cardiovascular complications, *25*, 95-99.

- (13) Malinow, M. R.; Bostom, A. G.; Krauss, R. M. *Circulation* **1999**, Homocyst(e)ine, diet, and cardiovascular diseases: A statement for health care professionals from the Nutrition Committee, American Heart Association, *99*, 178-182.
- (14) Graham, I. M.; Daly, L. E.; Refsum, H. M.; Robinson, K.; Brattstrom, L. E.; Ueland, P. M.; Palma-Reis, R. J.; Boers, G. H.; Sheahan, R. G.; Israelsson, B.; Uiterwaal, C. S.; Meleady, R.; McMaster, D.; Verhoef, P.; Witteman, J.; Rubba, P.; Bellet, H.; Wautrecht, J. C.; de Valk, H. W.; Sales Luis, A. C.; Parrot-Rouland, F. M.; Tan, K. S.; Higgins, I.; Garcon, D.; Andria, G. *Journal of the American Medical Association* **1997**, Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project, *277*, 1775-1781.
- (15) Waditee, R.; Bhuiyan Md Nazmul, H.; Rai, V.; Aoki, K.; Tanaka, Y.; Hibino, T.; Suzuki, S.; Takano, J.; Jagendorf Andre, T.; Takabe, T.; Takabe, T. *Proceedings of the National Academy of Sciences of the United States of America* **2005**, Genes for direct methylation of glycine provide high levels of glycinebetaine and abiotic-stress tolerance in *Synechococcus* and *Arabidopsis*, *102*, 1318-1323.
- (16) Nyyssola, A.; Kerovuuo, J.; Kaukinen, P.; von Weymarn, N.; Reinikainen, T. *Journal of Biological Chemistry*, Extreme halophiles synthesize betaine from glycine by methylation, *275*, 22196-22201.
- (17) Kunin, C. M.; Hua, T. H.; Van Arsdale White, L.; Villarejo, M. *Journal of Infectious Diseases*, Growth of *Escherichia coli* in human urine: role of salt tolerance and accumulation of glycine betaine, *166*, 1311-1315.
- (18) Lang, F.; Busch, G. L.; Voelkl, H. *Cellular Physiology and Biochemistry* **1998**, The diversity of volume regulatory mechanisms, *8*, 1-45.
- (19) Anjum, F.; Rishi, V.; Ahmad, F. *Biochimica et Biophysica Acta* **2000**, Compatibility of osmolytes with Gibbs energy of stabilization of proteins, *1476*, 75-84.
- (20) Knapp, S.; Ladenstein, R.; Galinski, E. A. *Extremophiles* **1999**, Extrinsic protein stabilization by the naturally occurring osmolytes b-hydroxyectoine and betaine, *3*, 191-198.
- (21) Moelbert, S.; Normand, B.; De Los Rios, P. *Biophysical Chemistry* **2004**, Kosmotropes and chaotropes: modelling preferential exclusion, binding and aggregate stability, *112*, 45-57.



- (22) Maclagan, R. G. A. R.; Malardier-Jugroot, C.; Whitehead, M. A.; Lever, M. *Journal of Physical Chemistry A* **2004**, Theoretical Studies of the Interaction of Water with Compensatory and Noncompensatory Solutes for Proteins, *108*, 2514-2519.
- (23) Selhub, J. *Annual Review of Nutrition* **1999**, Homocysteine metabolism, *19*, 217-246.
- (24) de Zwart, F. J.; Slow, S.; Payne, R. J.; Lever, M.; George, P. M.; Gerrard, J. A.; Chambers, S. T. *Food Chemistry* **2003**, Glycine betaine and glycine betaine analogues in common foods, *83*, 197-204.
- (25) Barak, A. J.; Beckenhauer, H. C.; Tuma, D. J. *Alcohol (New York, NY, United States)* **2002**, Methionine synthase: A possible prime site of the ethanolic lesion in liver, *26*, 65-67.
- (26) Sparks, J. D.; Collins, H. L.; Chirieac, D. V.; Ciani, J.; Jokinen, J.; Sowden, M. P.; Galloway, C. A.; Sparks, C. E. *Biochemical Journal* **2006**, Hepatic very-low-density lipoprotein and apolipoprotein B production are increased following in vivo induction of betaine-homocysteine S-methyltransferase, *395*, 363-371.
- (27) Haubrich, D. R.; Gerber, N. H. *Biochemical Pharmacology* **1981**, Choline dehydrogenase. Assay, properties and inhibitors, *30*, 2993-3000.
- (28) Meskys, R.; Harris, R. J.; Casaite, V.; Basran, J.; Scrutton, N. S. *European Journal of Biochemistry* **2001**, Organization of the genes involved in dimethylglycine and sarcosine degradation in *Artherobacter* spp. Implications for glycine betaine catabolism, *268*, 3390-3398.
- (29) Finkelstein, J. D.; Martin, J. J. *Journal of Biological Chemistry* **1984**, Methionine metabolism in mammals. Distribution of homocysteine between competing pathways, *259*, 9508-9513.
- (30) Rossi, E.; Beilby, J. P.; McQuillan, B. M.; Hung, J. *Annals of Clinical Biochemistry* **1999**, Biological variability and reference intervals for total plasma homocysteine, *36*, 56-61.
- (31) Schreiner, R.; Göbel-Schreiner, B.; Durst, C.; Casper, R.; Walch, S. *Clinical Laboratory* **1997**, Homocysteine: reference values, *43*.
- (32) Weisberg, I. S.; Park, E.; Ballman, K. V.; Berger, P.; Nunn, M.; Suh, D. S.; Breksa, A. P., III; Garrow, T. A.; Rozen, R. *Atherosclerosis* **2003**, Investigations of a common genetic variant in betaine-homocysteine methyltransferase (BHMT) in coronary artery disease, *167*, 205-214.
- (33) Christensen, B.; Refsum, H.; Vintermyr, O.; Ueland, P. M. *Journal of Cellular Physiology* **1991**, Homocysteine export from cells cultured in the presence of physiological or

- superfluous levels of methionine: methionine loading of non-transformed, transformed, proliferating, and quiescent cells in culture, *146*, 52-62.
- (34) Welch, G. N.; Loscalzo, J. *New England Journal of Medicine* **1998**, Homocysteine and atherothrombosis, *338*, 1042-1050.
- (35) Lever, M.; Sizeland, P. C. M.; Frampton, C. M.; Chambers, S. T. *Clinical Biochemistry* **2004**, Short and long-term variation of plasma glycine betaine concentrations in humans.
- (36) Stamler, J. S.; Osborne, J. A.; Jaraki, O.; Rabbani, L. E.; Mullins, M.; Singel, D.; Loscalzo, J. *Journal of Clinical Investigation* **1993**, Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen, *91*, 308-318.
- (37) Garrow, T. A. *Journal of Biological Chemistry* **1996**, Purification, kinetic properties, and cDNA cloning of mammalian betaine-homocysteine methyltransferase, *271*, 22831-22838.
- (38) Lee, K. H.; Cava, M.; Amiri, P.; Ottoboni, T.; Lindquist, R. N. *Archives of Biochemistry and Biophysics* **1992**, Betaine:homocysteine methyltransferase from rat liver: purification and inhibition by a boronic acid substrate analog, *292*, 77-86.
- (39) Skiba, W. E.; Wells, M. S.; Mangum, J. H.; Awad, W. M., Jr. *Methods in Enzymology* **1987**, Betaine-homocysteine S-methyltransferase (human), *143*, 384-388.
- (40) Ericson, L. E. *Acta Chemica Scandinavica* **1960**, Betaine-homocysteine methyltransferases. II. Isolation and properties of the transferase of pig liver, *14*, 2113-2126.
- (41) Evans, J. C.; Huddler, D. P.; Jiracek, J.; Castro, C.; Millian, N. S.; Garrow, T. A.; Ludwig, M. L. *Structure* **2002**, Betaine-Homocysteine Methyltransferase Zinc in a Distorted Barrel, *10*, 1159-1171.
- (42) Gonzalez, B.; Pajares, M. A.; Martinez-Ripoll, M.; Blundell, T. L.; Sanz-Aparicio, J. *Journal of Molecular Biology* **2004**, Crystal Structure of Rat Liver Betaine Homocysteine S-Methyltransferase Reveals New Oligomerization Features and Conformational Changes Upon Substrate Binding, *338*, 771-782.
- (43) Millian, N. S.; Garrow, T. A. *Archives of Biochemistry and Biophysics* **1998**, Human betaine-homocysteine methyltransferase is a zinc metalloenzyme, *356*, 93-98.

- (44) Breksa, A. P., III; Garrow, T. A. *Biochemistry* **1999**, Recombinant human liver betaine-homocysteine S-methyltransferase: Identification of three cysteine residues critical for zinc binding., *38*, 13991-13998.
- (45) Awad, W. M., Jr.; Whitney, P. L.; Skiba, W. E.; Mangum, J. H.; Wells, M. S. *Journal of Biological Chemistry* **1983**, Evidence for direct methyl transfer in betaine:homocysteine S-methyltransferase, *258*, 12790-12792.
- (46) Castro, C.; Gratson, A. A.; Evans, J. C.; Jiracek, J.; Collinsova, M.; Ludwig, M. L.; Garrow, T. A. *Biochemistry* **2004**, Dissecting the Catalytic Mechanism of Betaine-Homocysteine S-Methyltransferase by Use of Intrinsic Tryptophan Fluorescence and Site-Directed Mutagenesis, *43*, 5341-5351.
- (47) Vivian, J. T.; Callis, P. R. *Biophysical Journal* **2001**, Mechanisms of tryptophan fluorescence shifts in proteins, *80*, 2093-2109.
- (48) Sowden, M. P.; Collins, H. L.; Smith, H. C.; Garrow, T. A.; Sparks, J. D.; Sparks, C. E. *Biochemical Journal* **1999**, Apolipoprotein B mRNA and lipoprotein secretion are increased in McArdle RH-7777 cells by expression of betaine-homocysteine S-methyltransferase, *341*, 639-645.
- (49) Balinska, M.; Paszewski, A. *Biochemical and Biophysical Research Communications* **1979**, Betaine-homocysteine methyltransferase in the fungus *Aspergillus nidulans*, *91*, 1095-1100.
- (50) Pichereau, V.; Cosquer, A.; Gaumont, A.-C.; Bernard, T. *Bioorganic & Medicinal Chemistry Letters* **1997**, Synthesis of trimethylated phosphonium and arsonium analogs of the osmoprotectant glycine betaine; contrasted biological activities in two bacterial species, *7*, 2893-2896.
- (51) Ericson, L. E. *Acta Chemica Scandinavica* **1960**, Betaine-homocysteine methyltransferases. I. Distribution in nature, *14*, 2102-2112.
- (52) Maw, G. A. *Biochemical Journal* **1959**, Thetin-homocysteine transmethylase. The distribution of the enzyme, studied with the aid of trimethylsulfonium chloride as substrate, *72*, 602-608.
- (53) Sunden, S. L. F.; Renduchintala, M. S.; Park, E. I.; Miklasz, S. D.; Garrow, T. A. *Archives of Biochemistry and Biophysics* **1997**, Betaine-homocysteine methyltransferase expression in porcine and human tissues and chromosomal localization of the human gene, *345*, 171-174.

- (54) Rao, P. V.; Garrow, T. A.; John, F.; Garland, D.; Millian, N. S.; Zigler, J. S., Jr. *Journal of Biological Chemistry* **1998**, Betaine-homocysteine methyltransferase is a developmentally regulated enzyme crystallin in rhesus monkey lens, *273*, 30669-30674.
- (55) Xue, G. P.; Snoswell, A. M. *Comparative Biochemistry and Physiology, Part B: Biochemistry & Molecular Biology* **1986**, Developmental changes in the activities of enzymes related to methyl group metabolism in sheep tissues, *83B*, 115-120.
- (56) Gaull, G. E.; Von Berg, W.; Raiha, N. C.; Sturman, J. A. *Pediatric Research* **1973**, Development of methyltransferase activities of human fetal tissues, *7*, 527-533.
- (57) Matthews, R. G.; Smith, A. E.; Zhou, Z. S.; Taurog, R. E.; Bandarian, V.; Evans, J. C.; Ludwig, M. *Helvetica Chimica Acta* **2003**, Cobalamin-dependent and cobalamin-independent methionine synthases: Are there two solutions to the same chemical problem?, *86*, 3939-3954.
- (58) Smith, A. E.; Matthews, R. G. *Biochemistry* **2000**, Protonation state of methyltetrahydrofolate in a binary complex with cobalamin-dependent methionine synthase, *39*, 13880-13890.
- (59) Benesch, R. E.; Lardy, H. A.; Benesch, R. *Journal of Biological Chemistry* **1955**, The sulfhydryl groups of crystalline proteins. I. Some albumins, enzymes, and hemoglobins, *216*, 663-676.
- (60) Kacprzak, M. M.; Lewandowska, I.; Matthews, R. G.; Paszewski, A. *Biochemical Journal* **2003**, Transcriptional regulation of methionine synthase by homocysteine and choline in *Aspergillus nidulans*, *376*, 517-524.
- (61) Park, E. I.; Garrow, T. A. *Journal of Biological Chemistry* **1999**, Interaction between dietary methionine and methyl donor intake on rat liver betaine-homocysteine methyltransferase gene expression and organization of the human gene., *274*, 7816-7824.
- (62) Heil, S. G.; Lievers, K. J. A.; Boers, G. H.; Verhoef, P.; den Heijer, M.; Trijbels, F. J. M.; Blom, H. J. *Molecular Genetics and Metabolism* **2000**, Betaine-Homocysteine Methyltransferase (BHMT): Genomic Sequencing and Relevance to Hyperhomocysteinemia and Vascular Disease in Humans., *71*, 511-519.
- (63) Chadwick, L. H.; McCandless, S. E.; Silverman, G. L.; Schwartz, S.; Westaway, D.; Nadeau, J. H. *Genomics* **2000**, Betaine-homocysteine methyltransferase-2: cDNA

- cloning, gene sequence, physical mapping, and expression of the human and mouse genes., *70*, 66-73.
- (64) Dinning, J. S.; Neatrou, R.; Day, P. L. *Proceedings of the Society for Experimental Biology and Medicine* **1953**, Effect of diet on rat-liver betaine transmethylase, *84*, 499-501.
- (65) Ericson, L. E.; Harper, A. E. *Journal of Biological Chemistry* **1956**, Effect of diet on the betaine-homocysteine transmethylase activity of rat liver. I. Amino acids and protein, *219*, 49-58.
- (66) Finkelstein, J. D.; Kyle, W.; Harris, B. J. *Archives of Biochemistry and Biophysics* **1971**, Methionine metabolism in mammals. Regulation of homocysteine methyltransferases in rat tissue, *146*, 84-92.
- (67) Finkelstein, J. D.; Harris, B. J.; Martin, J. J.; Kyle, W. E. *Biochemical and Biophysical Research Communications* **1982**, Regulation of hepatic betaine-homocysteine methyltransferase by dietary methionine, *108*, 344-348.
- (68) Finkelstein, J. D.; Martin, J. J.; Harris, B. J.; Kyle, W. E. *Journal of Nutrition* **1983**, Regulation of hepatic betaine-homocysteine methyltransferase by dietary betaine, *113*, 519-521.
- (69) Emmert, J. L.; Garrow, T. A.; Baker, D. H. *Journal of Nutrition* **1996**, Hepatic betaine-homocysteine methyltransferase activity in the chicken is influenced by dietary intake of sulfur amino acids, choline and betaine., *126*, 2050-2058.
- (70) De Oliveira, A. C.; D'Almeida, V.; Hipolide, D. C.; Nobrega, J. N.; Tufik, S. *Canadian Journal of Physiology and Pharmacology* **2002**, Sleep deprivation reduces total plasma homocysteine levels in rats, *80*, 193-197.
- (71) Yamauchi, A.; Uchida, S.; Kwon, H. M.; Preston, A. S.; Robey, R. B.; Garcia-Perez, A.; Burg, M. B.; Handler, J. S. *Journal of Biological Chemistry* **1992**, Cloning of a Na(+)- and Cl(-)-dependent betaine transporter that is regulated by hypertonicity, *267*, 649-652.
- (72) Grossman, E. B.; Hebert, S. C. *American Journal of Physiology* **1989**, Renal inner medullary choline dehydrogenase activity: characterization and modulation, *256*, F107-112.
- (73) Delgado-Reyes, C. V.; Garrow, T. A. *American Journal of Physiology* **2005**, High sodium chloride intake decreases betaine-homocysteine S-methyltransferase expression in guinea pig liver and kidney, *288*, R182-R187.

- (74) Harper, A. E.; Ericson, L. E.; Boldt, R. E.; Elvehjem, C. A. *American Journal of Physiology* **1956**, Effect of thyroid-active substances on the betaine-homocysteine transmethylase activity of rat liver, *184*, 457-460.
- (75) Shibata, T.; Akamine, T.; Nikki, T.; Yamashita, H.; Nobukuni, K. *Poultry Science* **2003**, Synthesis of betaine-homocysteine S-methyltransferase is continuously enhanced in fatty livers of thyroidectomized chickens, *82*, 207-213.
- (76) Wijekoon, E. P.; Hall, B.; Ratnam, S.; Brosnan, M. E.; Zeisel, S. H.; Brosnan, J. T. *Diabetes* **2005**, Homocysteine metabolism in ZDF (type 2) diabetic rats, *54*, 3245-3251.
- (77) Ratnam, S.; Wijekoon, E. P.; Hall, B.; Garrow, T. A.; Brosnan, M. E.; Brosnan, J. T. *American Journal of Physiology* **2006**, Effects of diabetes and insulin on betaine-homocysteine S-methyltransferase expression in rat liver, *290*, E933-E939.
- (78) Martin, J. J.; Finkelstein, J. D. *Analytical Biochemistry* **1981**, Enzymic determination of betaine in rat tissues, *111*, 72-76.
- (79) Mudd, S. H.; Finkelstein, J. D.; Irreverre, F.; Laster, L. *Journal of Biological Chemistry* **1965**, Transsulfuration in mammals. Microassays and tissue distributions of three enzymes of the pathway, *240*, 4382-4392.
- (80) Slow, S.; Donaggio, M.; Cressey, P. J.; Lever, M.; George, P. M.; Chambers, S. T. *Journal of Food Composition and Analysis* **2005**, The betaine content of New Zealand foods and estimated intake in the New Zealand diet, *18*, 473-485.
- (81) Lever, M.; Bason, L.; Leaver, C.; Hayman, C. M.; Chambers, S. T. *Analytical Biochemistry* **1992**, Same-day batch measurement of glycine betaine, carnitine, and other betaines in biological material, *205*, 14-21.
- (82) Lever, M.; Sizeland, P. C. B.; Bason, L. M.; Hayman, C. M.; Chambers, S. T. *Biochimica et Biophysica Acta* **1994**, Glycine betaine and proline betaine in human blood and urine, *1200*, 259-264.
- (83) Mulligan, J. D.; Laurie, T. J.; Garrow, T. A. *Journal of Nutritional Biochemistry* **1998**, An assay for betaine-homocysteine methyltransferase activity based on the microbial detection of methionine, *9*, 351-354.
- (84) Chambers, S. T.; Lever, M. *Nephron* **1996**, Betaines and urinary tract infections, *74*, 1-10.

Chapter 2

# Protein Purification

## CHAPTER 2: Protein Purification

### 2.1 Introduction

In order to study a protein properly, it is desirable to purify it first. However, purification of a protein from tissue is often a laborious process, and is difficult to scale up. If more protein is required, often there is a trade-off between purity and yield, a more pure protein often results in less activity through the denaturation of proteins from mechanical and chemical stresses that are involved in the purification steps.

The type of study anticipated for the enzyme dictates the purity required. If detection of activity is all that is required, then analysis of crude extracts may be sufficient to give useful information. For structure-function studies, high degrees of purity may be needed to remove interference from other proteins. Activity studies do not require high purity, however, all conflicting activities must be removed to ensure that the activity measured is the activity of interest. Also, a rapid purification is desirable as prolonged purifications often yield relatively low activities as proteins denature.

The amount of purified protein obtained will not only depend on the starting material, but also the efficiency of the purification steps. Purification steps must be carefully selected to give the best yields, while providing significant enrichment and rapid throughput. Where a choice exists, the starting material that will give the fewest contaminants, while allowing the easiest separation and greatest yields should be chosen.

BHMT is not widely distributed in animals; it is found almost solely in the liver, which reduces the options for tissues to start purification. Recombinant human and rat BHMT have been produced by Breska *et al.*<sup>1</sup> and have proven to be useful in structure analysis (both



structures have been solved from X-ray crystal data),<sup>2,3</sup> but may lack post-translational modifications found in the native mammalian liver enzyme.

Specifically, for the studies described in this thesis, BHMT was purified from liver, using acetone precipitation, thermal stability, anionic exchange and molecular mass. This chapter outlines, individually, the steps taken during each purification step, and then brings them together into a purification protocol [Section 2.6]. Finally, mass spectrometry and *N*-terminal sequencing of the BHMT subunit, conclusively showed the presence of BHMT in the solution.

### 2.2 Sources of Protein

BHMT was extracted primarily from rat and sheep liver. These tissues were selected for several reasons. Most mammals express BHMT in the liver and kidney, and BHMT makes up to 1.6% of total soluble liver protein.<sup>4</sup> Rat and sheep liver is also relatively easy to obtain and can be easily homogenised.

Initially rat livers were washed in 20 mM potassium phosphate buffer at pH 7.5. This effectively removes much of the haemoglobin, which can complicate separation of BHMT. The liver was then homogenised using a turreted homogeniser (5000 rpm) at room temperature for 20 s with two volumes of phosphate buffer. The resulting suspension was centrifuged (10,000 g) for 30 min at 4 °C to remove the cellular debris. The resulting supernatant contains BHMT activity, measured by adding 0.1 mL of crude homogenate with 0.1 mL of 10 mM glycine betaine, and 0.1 mL of 10 mM homocysteine (preparation described in **Chapter 4**) and 0.2 mL of 20 mM potassium phosphate buffer at pH 7.5. The sample was then assayed using the NMR assay in **Chapter 4**, and activity identified by the production of methionine.

## 2.3 Heat Denaturation

### 2.3.1 Introduction

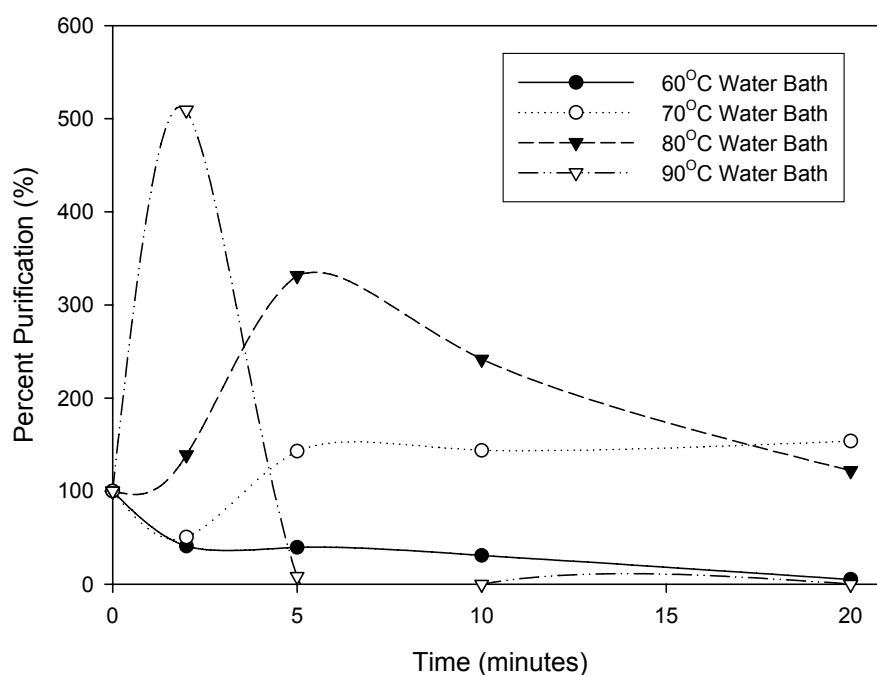
BHMT is a thermostable protein, capable of withstanding high temperatures (70 °C) as initially described by Durell in 1957, when working on “thetin homocysteine methylpherase”.<sup>5</sup> Heat denaturation, therefore, is an effective purification step as most other proteins are denatured at temperatures above 50 °C for very short times. Durell heated liver homogenate to 70 °C for 5 min, BHMT is stable for up to 10 min at these temperatures without denaturing.<sup>5-9</sup> Eppendorf tubes with a 1 cm bore were used to ensure rapid heating once plunged into a hot water bath and rapid cooling of the resulting protein solution.

It should be noted that this procedure is favoured in the literature.<sup>5-9</sup> Almost all BHMT purifications begin with this heat denaturation step, although there are no data on optimisation of this purification step.

### 2.3.2 Method and Results

Rat liver homogenate (1 mL) was incubated in a pre-heated water bath at 60, 70, 80 and 90 °C, for 2, 5, 10 and 20 min. The homogenate was then plunged onto an ice/water slurry after the specified time. To ensure that the sample is heated in an approximately uniform manner, the heat shock was done in 1.5 mL Eppendorf tubes. The samples were then centrifuged (10 min, 4 °C at 10,000 g) and the soluble protein was analysed for BHMT activity at saturating concentrations of betaine and homocysteine (5 mM) using the NMR spectroscopic assay described in **Chapter 4**. The results are summarised in **Figure 2.1** below. Protein concentration was determined using an automated Bradford assay standardised against bovine serum albumin (BSA).

This experiment showed clearly that the literature preparation consisting of a 70 °C/10 min heat denaturation, was effective, but not optimal: heating at 90 °C for 2 min was over five times as efficient as a purification step, compared with the procedures of Durell.<sup>5</sup>



**Figure 2.1** Temperature dependence and time course of the heat denaturation step during BHMT purification. Purification is expressed as specific activity versus crude homogenate.

## 2.4 Anion-Exchange Chromatography

### 2.4.1 Introduction

Following heat denaturation, a range of chromatographic techniques were found to be available to further purify the protein mixture to homogeneity. Anion-exchange chromatography is a commonly used technique, based on the competitive binding of negatively charged species to a cationic resin. The resin was prepared and equilibrated with a buffer containing an anion, which was bound to the resin. Upon loading the heat denatured liver

extract, the most highly anionic species will competitively bind to the resin, displacing the buffer anion, and becoming immobile. After eluting the non-binding species from the column, a salt gradient (normally sodium chloride) was applied, competing with the anionic protein for the cationic centres on the resin and resulting in the elution of the anionic protein into the buffer.

Once removed from the column, the protein solution was desalted by dialysis or selective filtration (gel filtration or ultra filtration were used to de-salt the mixture). With respect to BHMT purification, the heat-denatured protein mixture produced was applied to the anion-exchange columns after adjusting the pH of the buffer towards a more basic solution (pH ~ 8.5-9).

### 2.4.2 Method

DEAE anion exchange resin (Pharmacia Biotech, A-50, 15 mL) was prepared by equilibration with TRIS buffer (50 mM, pH 9) and was loaded into a short column (30 cm x 1.7 cm diameter). The heat-treated liver extract (pH 9, 15 mL) was loaded onto the resin. The column was eluted with a linear sodium chloride gradient in TRIS buffer to 1 M NaCl (1 mL/min), with fractions being collected automatically every 10 mL and the  $UV_{280}$  absorbance being used as an indication of protein concentration. BHMT activity was detected by spiking the fractions to 5 mM glycine betaine and homocysteine, and NMR spectroscopy was used to detect the production of methionine [Chapter 4].

### 2.4.3 Results

The BHMT activity eluted as one peak, over the entire gradient as applied. Furthermore, the yield of BHMT was unclear, as the resulting BHMT activity appeared to be unstable. The activity of fractions deteriorated and after 48 h the activity had diminished to undetectable levels. There were a number of proteins that remained on the column,

observable from the strong discoloration of the column which remained after the salt gradient had been applied. The remaining protein could be removed using 2 M NaCl, giving a brown solution that contained no BHMT activity.

## 2.5 Size Exclusion Chromatography

### 2.5.1 Introduction

Size exclusion chromatography (gel filtration) is a commonly employed technique which separates biological molecules on the basis of size. It is useful for de-salting protein mixtures and for the crude separation of proteins with disparate molecular weights. The resins used for gel filtration are based on sugar (cellulose) backbones (Sephacrose) or synthetic equivalents (Sephadex, Superdex) and there are a variety of fractionation ranges available.

Like all forms of column chromatography the basis of separation is a difference between the target protein and the mobile/stationary phases. In gel filtration, the resin beads have well defined pores, which increase the volume of the mobile phase which is accessible to the proteins which are able to fit within the pores. Effectively, this means that proteins which are too large to be included within the resin pass through a relatively small elution volume ( $V_e$ ), whereas small proteins are eluted in a much larger volume by virtue of the extra eluant required to flush out the pores in the resin.

The pores in the resin fall within a range of sizes, enabling fractionation of mixtures of proteins of varying sizes. This also offers a technique for experimentally determining the molecular weight of the protein in question. By measuring the volume of buffer required to elute the unknown protein and comparing this to appropriate standards, a measure of the approximate size of the unknown can be obtained. This requires calibration of the column including a measure of  $V_0$ , the void volume, the smallest possible elution volume from the

column.  $V_0$  can be determined by applying a solution of blue dextran ( $M_r \sim 2000$  kDa), a dyed polysaccharide which gives an obvious chromatographic signal when it emerges from the column.

The elution volume can also be affected by other physical properties, such as protein shape. In general, long thin proteins will elute faster (in smaller volumes) than globular proteins. Gel filtration media have a varying fractionation range, and for the purposes of separating BHMT from other proteins and macromolecules in the heat denatured liver homogenates, G-200 was found to be most suitable (fractionation range of 10 kDa to 800 kDa for globular proteins).

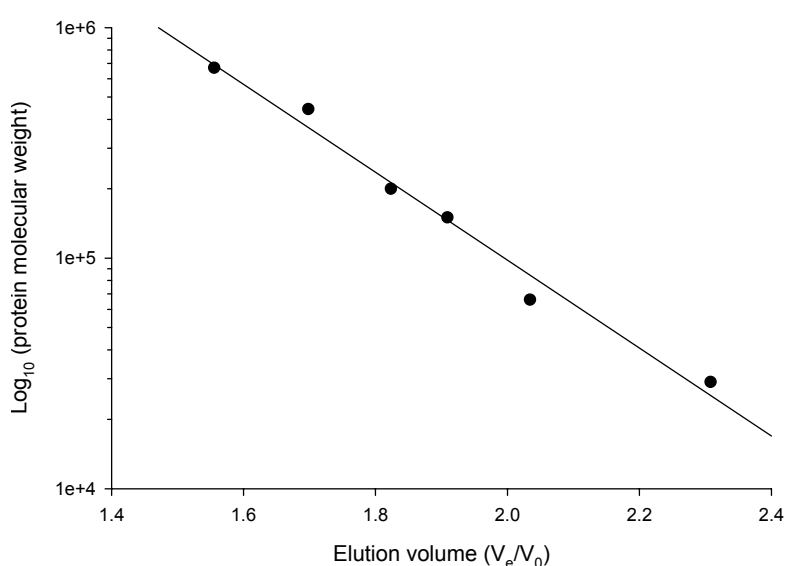
### 2.5.2 Method

600 mL of Sepharose CL-6B size exclusion resin (Sigma, CL-6B-200) was rinsed four times with 20 mM sodium phosphate buffer (800 mL at pH 7.5) and packed into a 2 m column under slight positive pressure using a peristaltic pump (1 mL/min). Once packed, the resin was flushed with 1 L of phosphate buffer and 2 mL of 1 mg/mL blue dextran was applied to the column. Phosphate buffer was applied using a peristaltic pump (flow rate 2 mL/min), and the eluant volume recorded until the blue dextran fraction was at its maximum concentration, as determined by UV absorbance (280 nm).

Protein standards (Sigma, MW-GF-1000) were applied following the manufacturer's instructions and their elution volumes recorded using UV absorbance (280 nm) under identical conditions to the blue dextran. The elution volume was expressed as a fraction of the void volume, and found to vary linearly with  $\log_{10}$  (protein size) [Figure 2.2].

To purify and estimate the molecular size of BHMT from liver, the heat denatured homogenate (15 mL) was applied gently to the column under gravity and then eluted with phosphate buffer under the same conditions as the dextran and standards. Fractions were

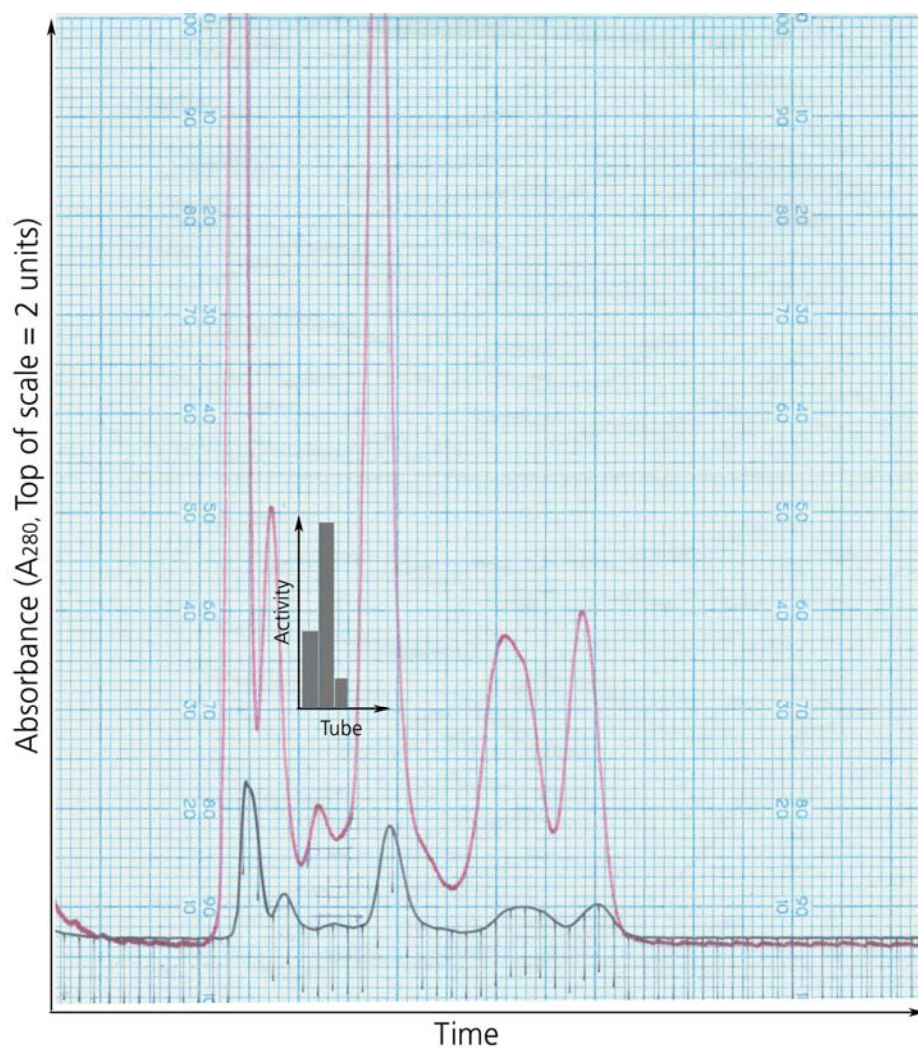
collected every 10 mL, and 2 mM glycine betaine and homocysteine were spiked into the fractions before NMR spectroscopy was used to assay for methionine production [Chapter 4]. Tubes with BHMT activity were pooled and concentrated using stirred concentration cells (Amersham Biosciences), and Bradford assays were used to determine the protein concentration of the pooled fractions.



**Figure 2.2** Calibration of G-200 column with standard molecular weight proteins.

### 2.5.3 Results

The calibration line for the Sepharose column is shown in **Figure 2.2**. The elution volume for the BHMT solutions, was 315.5 mL ( $V_e/V_0 = 1.79$ ) of buffer, that yielded a calculated size of  $241.3 \pm 4.8$  kDa (incorporating a 2% error in measurements estimated from uncertainties on measurements). There was also a notable peak of aggregated protein eluted at the void volume.



**Figure 2.3** Absorbance at 280 nm indicating protein elution from a G-200 column (flow rate, 1.1 mL/min, chart speed 0.2 mm/min). The activity of BHMT (inset), determined by  $^1\text{H}$ -NMR spectroscopy, was determined indicating the presence of BHMT (two bold squares indicate 10 'units' of BHMT activity or a DMG integral of 10 against 5 mM tBuOH by NMR spectroscopy) in the three active fractions.

## 2.6 Combined strategy

### 2.6.1 Introduction

After studying several steps as described in the sections above, a purification strategy was formulated including homogenisation, heat denaturation and size exclusion chromatography. Anion exchange chromatography was deliberately avoided, because the resulting fraction appeared to denature or lose activity slowly over a period of 48 h. Similarly,



TRIS buffer was avoided, because experience from anion exchange indicated that it appeared to reduce the activity of the enzyme.

## 2.6.2 Final Method

### 2.6.2.1 Crude Homogenate

Rat liver (24.3 g) was homogenised in 50 mL of potassium phosphate buffer (20 mM, pH~7.5). The resulting suspension was centrifuged at 10000 *g* and 4 °C for 30 min, as described above (**Section 2.2**). The resultant solution was assayed for protein concentration using the techniques described above, and a 100 µL aliquot was incubated for 24 h with 2 mM homocysteine and 2 mM glycine betaine, in the presence of a 5 mM *tert*-Butanol internal standard, as described in **Section 4.3**. The integral of methionine production (compared to the *tert*-Butanol integral) was used as an index of BHMT activity (an integral of 1 equating to one unit of activity). This measurement of activity was then divided by the total protein concentration to give the specific activity measurement.

### 2.6.2.2 Heat Denaturation

The homogenate from **Section 2.6.2.1** above (55 mL) was then divided into 1 mL Eppendorf tubes and plunged into a 90 °C water bath for 2 min. The tubes were placed immediately on ice for 5 min to reduce the temperature of the solution, then centrifuged at 4 °C for 10 min. The resultant solution was assayed for protein concentration using the techniques described above, and a 100 µL aliquot was incubated for 24 h with 2 mM homocysteine and 2 mM glycine betaine, in the presence of a 5 mM *tert*-butanol internal standard, as described in **Section 4.3**. The integral of methionine production (compared to the *tert*-Butanol integral) was used as an index of BHMT activity (an integral of 1 equating to one

unit of activity). This measurement of activity was then divided by the total protein concentration to give the specific activity measurement.

### *2.6.2.3 Size Exclusion Chromatography*

The clear homogenate obtained from **Section 2.6.2.2** above (31 mL) was then applied to a column, packed with SuperDex G200 (Sigma; ~450 mL resin, 2.6 cm x 83 cm) which had been washed with 1.5 L of potassium phosphate buffer (20 mM, pH~7.5). The column was eluted at 1.1 mL/min, with tube changes every 12 min. Protein concentration was monitored using absorbance at 280 nm. Fractions were spiked with 2 mM glycine betaine and homocysteine and incubated for 24 h. The fractions were then analysed using NMR spectroscopy. Those tubes in which DMG had been produced, and which indicated a dominant 45 kDa band by SDS-PAGE analysis were pooled and concentrated using a stirred concentration cell (Amersham Biosciences, 100 mL), washed through the concentration cell with potassium phosphate buffer (20 mM, pH~7.5) until no trace of methionine, glycine betaine, or DMG could be observed using NMR spectroscopy. The sample volume was reduced in the concentration cell under 40 psi to 10 mL. The resultant solution was assayed for protein concentration using the techniques described above [**Section 2.5.2**], and a 100  $\mu$ L aliquot was incubated for 24 h with 2 mM homocysteine and 2 mM glycine betaine, in the presence of a 5 mM *tert*-Butanol internal standard, as described in **Section 4.3**. The integral of methionine production (compared to the *tert*-butanol integral) was used as an index of BHMT activity (an integral of 1 equating to one unit of activity). This measurement of activity was then divided by the total protein concentration to give the specific activity measurement.

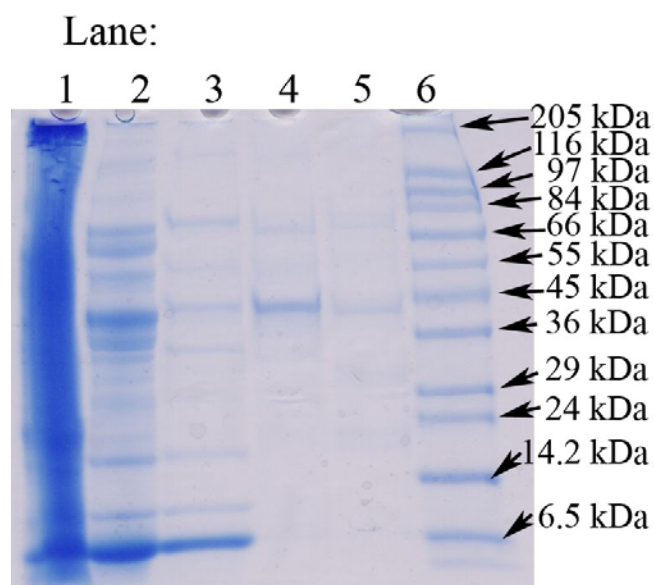
#### 2.6.2.4 SDS-PAGE Analysis

All SDS-PAGE analysis was conducted using Life Therapeutics 'Lifegels' (4-20% gradient) in Tris-HEPES-SDS buffer following the instructions provided in the kitset. Gels were refrigerated at 4 °C and 150 V was applied along the gel. Staining with Coomassie Blue and destaining were also performed using the instructions provided.

### 2.6.3 Results

Table 2.1 Purification Table:

Purification step	Protein Concentration (mg/mL)	Volume obtained (mL)	Activity (Units/mL)	Specific Activity (Units/mg)	Percent Yield (%)	Purification
Tissue Homogenate	22.82	55	75.1	3.3	100	1
Heat Denaturation	1.99	31	47	23.6	62.6	7.2
Size Exclusion Chromatography	0.19	35.5	20.7	108.9	27.6	33.1



**Figure 2.4** SDS-PAGE analysis of rat liver subjected to the purification procedures described in Section 2.6, and stained with Coomassie Brilliant Blue. Lane 1: Crude extract, Lane 2: 90 °C heat treatment, Lanes 3, 4 and 5: Size Exclusion Chromatography following the three active fractions [Figure 2.3] as they were eluted from the column, and Lane 6: Sigma Wide SDS-PAGE markers. The ~45 kDa subunit of BHMT is clearly visible after size exclusion chromatography, and the relative activity of these three fractions approximates the intensity of the 45 kDa band.

## 2.7 Mass Spectrometry Analysis

### 2.7.1 Introduction

To verify the presence of BHMT and to confirm that the observed activity was due to BHMT protein, a mass spectrum of the final purified BHMT solution was obtained. Mass spectrometry gives a total mass of the subunits present in the reaction mixture, and would also identify if there were any post-translational modifications of BHMT, including glycosylation (which would increase the mass of the BHMT subunit) and cleavage of the protein (which would give a decreased mass).

### 2.7.2 Method

A sample of the BHMT (0.8 mg/mL, 2 mL), purified as described in **Section 2.6** was de-salted against distilled water (3 x 500 mL) using dialysis at 4 °C for 8 h each exchange. The resultant solution was submitted for electrospray mass spectral analysis with Professor Stephen Brennan in the Christchurch School of Medicine.

Urea was added to the sample to 8 M and the sample was heated to 90 °C for 30 min to denature the protein. Iodoacetamide (150 µL, 55 mM) was added to block the cysteine residues.

The sample was then analysed directly by electrospray ionisation (ESI) mass spectrometry (MS) on a Platform II quadrupole analyser (Micromass). Injections of 10-20 µL were introduced to the ion source at 5 µL/min. The probe was charged at +3500 volts and the source maintained at 60 °C. The mass range 400–1200 m/z was scanned every 3 s with a cone voltage ramp of 30–60 volts and up to 80 scans were averaged in acquiring the raw data. Calibration was made over this same m/z range using the charge series generated by human  $\alpha$  globin and data was acquired and processed using MassLynx software and transformed onto a true molecular mass scale using maximum entropy (MaxEnt) software supplied with the instrument.

#### *2.7.2.1 Tryptic Digest of BHMT Subunit*

Tryptic digests were prepared from approximately 0.25 mg of the white precipitate BHMT as described in the mass spectral work above. The individual chains were dried under N<sub>2</sub> and redissolved in 50 µL of 50 mM NH<sub>4</sub>HCO<sub>3</sub>. Trypsin (1.5 µg) was added and the reaction

incubated for 16 h at 37 °C. After drying under vacuum with  $P_4O_{10}$ , digests were redissolved in 100  $\mu$ l of 0.1% HCOOH, 50% acetonitrile and 10  $\mu$ l was analyzed by ESI MS as above. The m/z range 300–1900 was scanned every 4 s.

### 2.7.3 Results

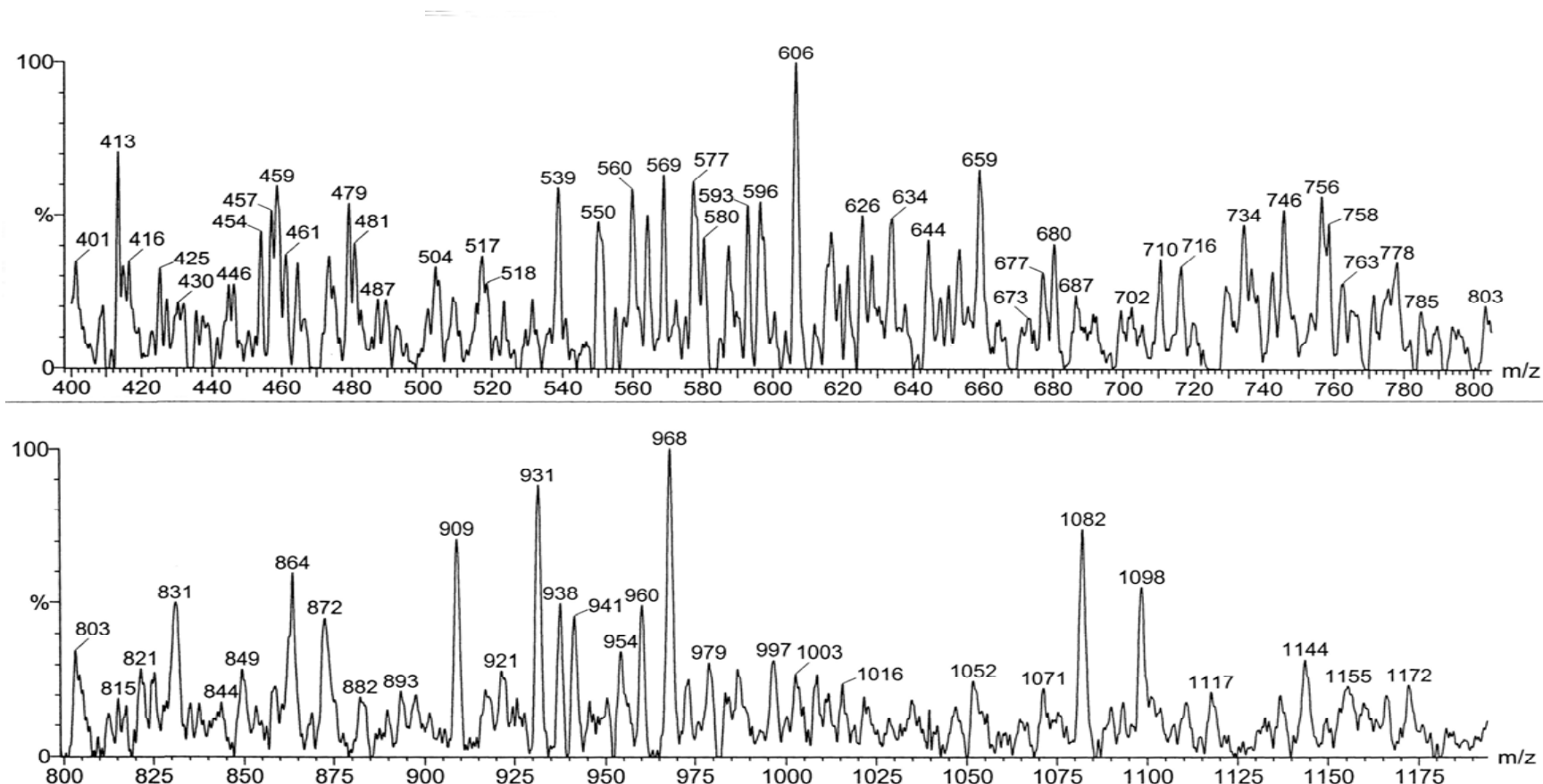
Initial attempts to obtain a mass of the entire BHMT subunit were unsuccessful. Trace amounts of albumin were identified in the mass spectra, but the expected BHMT subunit mass was not identified. It was noted, however, that small amounts of precipitate were forming at the bottom of the Eppendorf tubes following dialysis. Tryptic digest of the solution and these precipitates gave sufficient fragments to map 75% of the BHMT subunit, including two pairs of fragments which were also identified as conjoint sequences [Figure 2.4].



**Figure 2.5** Predicted rat BHMT amino acid sequence showing (marked) the observed tryptic fragments of BHMT, as identified by electrospray mass spectrometry. Cyan indicates the possible N-linked glycosylation site. These fragments are summarised in **Table 2.2** below.

**Table 2.2** Observed fragments from tryptic digest of BHMT subunit (complete listing of tryptic fragments is in **Appendix 1**).

Fragment	Fragment Sequence	Mass (Daltons)	Observed M/Z (3 s/f)
T5	GILER	586.34	587
T6	LNAGEVVIGDGGFVFALEK	1934.01	968
T9	AGPWTPEAAVEHPEAVR	1815.89	606, 909
T10/11	QLHREFLR	1115.62	530
T11	EFLR	563.31	564
T12/13	AGSNVMQTFYASEDKLENR	2425.12	803
T16	VNEAAJDIAR	1117.52	560, 1117
	VNEAAJDIARQVADEGDALVAGGVSQTPS		593
T16/17	YLSJK	3568.68	
T17/18	QVADEGDALVAGGVSQTPSYLSJKSETEVK	3412.47	625, 782, 1042
T20	IFHQQLEVFMK	1418.74	710
T22	NVDFLIAEYFEHVEEAVWAVEALK	2820.4	941
T23	TSGKPIAATMJIGPEGDLHGVSPGEJAVR	2966.4	476
T25	AGAAIVGVNJHFDPSLSLQTIK	2285.15	446, 558, 743
T27	EGLEAAR	744.38	745
	AYLMSHALAYHTPDJGKQGFIDLPEFPFGLE		756, 945
T29/30	PR	3794.82	
T34/35	EAYNLGVR YIGGJJGFEPYHIR	2648.04	658, 877
T35	YIGGJJGFEPYHIR	1727.75	577, 864
T36	AIAEELAPER	1097.57	550, 1098
T36/37	AIAEELAPERGFPPASEK	2042.07	676
T41/42	KEYWQNLRL	1153.59	569, 1140
T43	IASGRRPYNPSMSKPDAGVTK	2261.13	607
T45	EATTEQQLR	1074.53	538
T48/49	FKSAQ	597.31	580



**Figure 2.6** Mass spectrum of rat BHMT after tryptic digestion. Peaks are identified in **Table 2.2** above.



## 2.8 *N*-terminal Sequencing

### 2.8.1 Introduction

To confirm that the detected BHMT activity was due to the rat BHMT protein, *N*-terminal sequencing was conducted on the 45 kDa band seen in the SDS-PAGE gel, suspected to be the BHMT subunit, comparing it to the rat cDNA data available.

### 2.8.2 Method

Rat BHMT extract, obtained after size exclusion chromatography and concentrated to 1 mg/mL was analysed using gradient SDS-PAGE electrophoresis (5-20%, Sigma) and stained for 1 h with Coomassie Blue stain. The stain was removed using 10% acetic acid in methanol, washed twice for 2 h each time. A second SDS-PAGE was conducted using the same extract and markers, but was not stained. The second gel was electro-blotted at 100 mA constant current (voltage ~45 V) for 15 h to PVDF membrane and stained for 1 min with amido black stain, followed by washing twice with 10% acetic acid in water.

The 45 kDa band was identified on the PVDF membrane and excised. The sample was submitted to the Protein Microchemistry Facility in the Department of Biochemistry at the University of Otago, Dunedin, New Zealand. For the rat sequence only 10 amino acids were sequenced to identify BHMT. The same procedures were then performed on the hagfish liver extract, post size-exclusion chromatography, and concentration to 1 mg/mL. Sigma wide-range markers (M 4038, Sigma) were used to confirm and identify the ~45 kDa band in the SDS-PAGE and PVDF blot [Chapter 6].

### 2.8.3 Results

The Protein Microchemistry facility obtained the predicted Rat cDNA sequence of “A P I A G K K A K R G I L E R” [Figure 2.4] together with low yields of sequences with staggered

starting *N*-termini into the chain. Despite a clear dark 45 kDa band being excised in rat BHMT it gave lower yields than expected, approximately 30 pmol. There was evidence of amino peptidase activity cleaving *N*-terminal amino acids, giving a staggered starting point and making interpretation of the sequence data difficult.

## 2.9 Genomic Sequencing of Rat *BHMT*

### 2.9.1 Introduction

As the initial *N*-terminal protein sequence of rat BHMT was ambiguous, genomic DNA was extracted from two of the Sprague-Dawley rats and the DNA sequence that encodes this region [Figure 2.5] was determined.

### 2.9.2 Method

#### *6.5.2.1 Genomic DNA extraction*

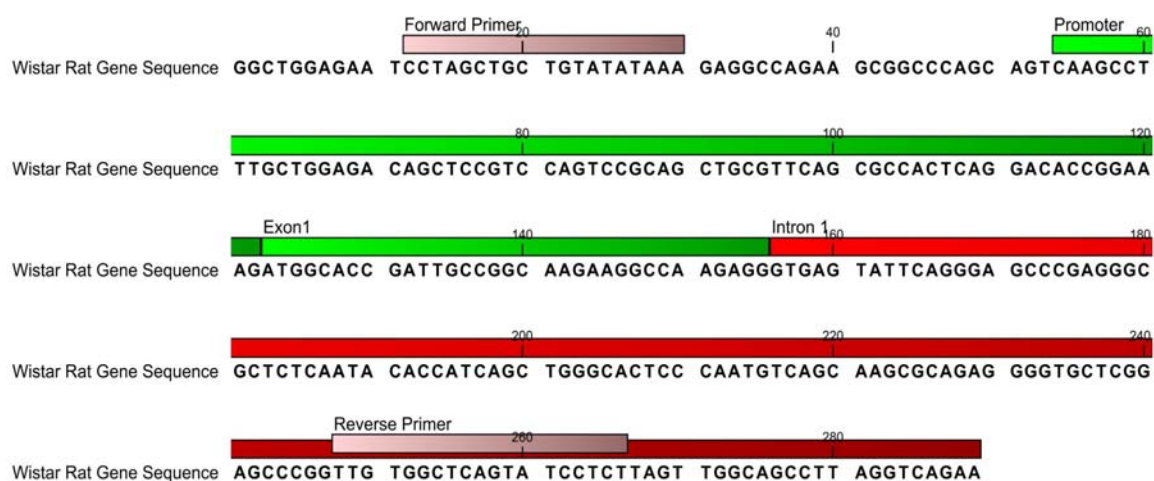
Tissue (0.5 g) was excised from the liver of two Sprague-Dawley rat livers which had been stored at -80 °C. Genomic DNA was purified using the reagents and procedures in the 'QIAamp DNA Mini Kit' (Qiagen, Valencia, CA 91355). The samples were suspended in 180 µL of 'ATL' buffer with 20 µL of 'protease K', mixed and incubated at 56 °C for 2.5 h with vortex stirring every 20 min. The sample was centrifuged briefly, then a further 200 µL 'ATL buffer' was added, the sample was mixed thoroughly and incubated for 10 min at 70 °C. The sample was then briefly centrifuged, and 200 µL of 100% ethanol was added, mixed and the sample was loaded onto a 'QIAamp Spin Column' and centrifuged at 6000 g for 1 min. The filtrate was discarded, and 500 µL of 'AW1 buffer' was applied to the column, followed by centrifugation at 6000 g for 1 min. The filtrate was discarded and 500 µL of 'AW2 buffer'

applied and centrifuged at 20000 g for 1 min. The filtrate was discarded and 200  $\mu$ L of TE buffer was applied, left to sit for 5 min, then centrifuged through the column at 6000 g for 1 min. DNA concentration was determined by absorbance at 280 nm and purity determined by the ratio of  $A_{260}/A_{280}$ .

#### 6.5.2.2 PCR Amplification of Exon-1/Intron-1 Boundary

PCR primers were designed and ordered through Invitrogen (Auckland, New Zealand) to correspond to the 5' region 40 bp upstream of Exon-1 and 120 bp downstream of the Exon-1/Intron-1 boundary [Figure 2.5]. The sequences selected were (forward) 5'-GGATCGACGACATATATT-3' and (reverse) 5'-AGAGGATACTGAGCCACAA-3'.

The primers were diluted to 50 ng/mL upon arrival and used at this concentration in all PCR procedures. PCR was conducted to amplify Exon-1 and the flanking region, using a 94 °C denaturation, followed by a 54-60 °C temperature gradient annealing and 72 °C polymerisation method, repeated for 32 cycles.



**Figure 2.7** Wistar rat *BHMT* genomic DNA sequence, showing the primer sequences ordered, and the Promotor, Exon1 and Intron1 region sequenced from the Sprague-Dawley rats.

### 2.9.2.3 Genomic Sequencing

Sequencing was performed using an Applied Biosystems '3130 XL' automatic sequencer using the Applied Biosystems 'BigDye' v3.1 Cycle Sequencing Kit.

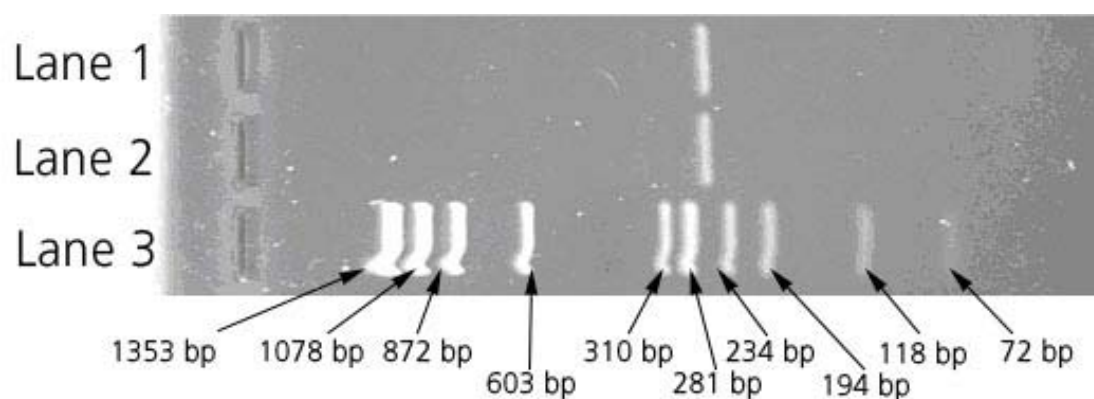
## 2.9.3 Results

### 2.9.3.1 DNA Extraction

DNA was obtained from both rats investigated and was of acceptable purity as determined by the  $A_{260}/A_{280}$  ratio (379 ng/ $\mu$ L for rat-1 and 640 ng/ $\mu$ L for rat-2,  $A_{260}/A_{280}$  1.93 and 1.95, respectively).

### 2.9.3.2 PCR Amplification

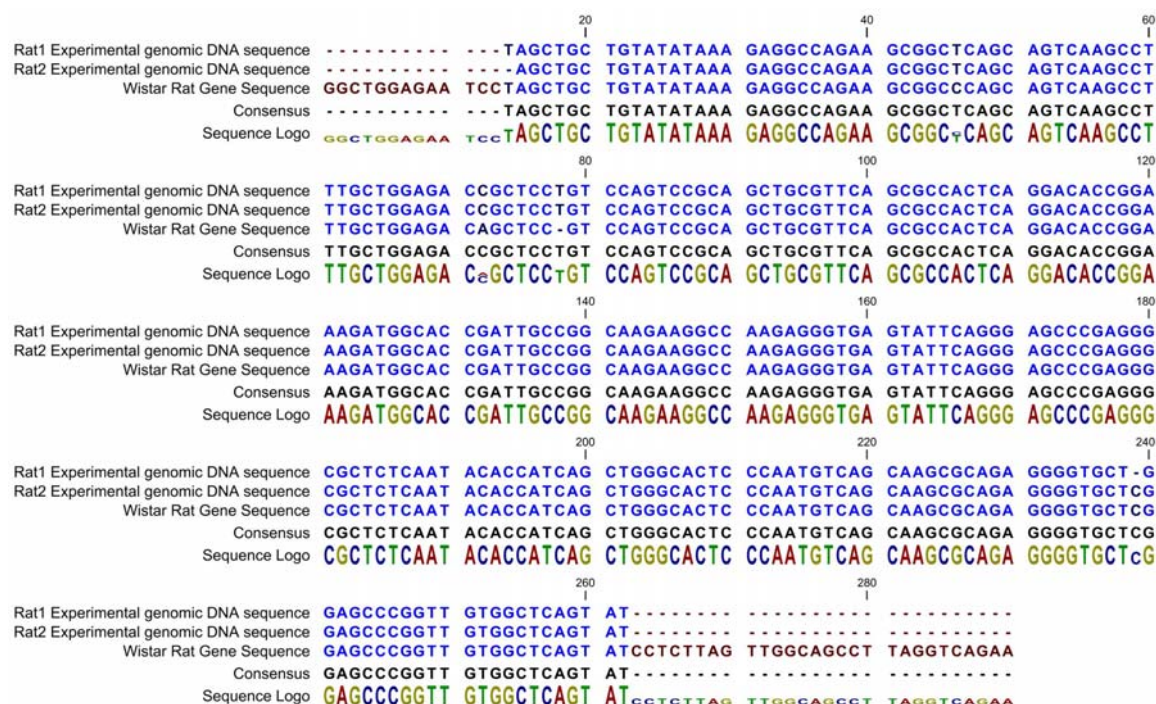
The product of PCR amplification of the rat genomic DNA was visualised using 2% agarose electrophoresis. PCR product was obtained in the PCR reactions which had been amplified at 54-58 °C. Electrophoresis analysis in 2% agarose at 100 V revealed the presence of the expected ~260 bp DNA fragment in both rat-1 and rat-2 [Figure 2.6].



**Figure 2.8** 2% agarose analysis of the PCR products. Lane 3 (bottom) contains Invitrogen 'PhiX 174 RF DNA/HaeIII' markers, lane 2 (middle) contains 5  $\mu$ L rat-1 DNA post PCR amplification and lane 1 (top) contains 5  $\mu$ L rat-2 DNA post PCR amplification. Each PCR product is 260 bp in size.

### 2.9.3.3 Sequence obtained

The sequence obtained is recorded in **Figure 2.7** and aligned with the genomic sequence obtained from the NCBI database of Wistar Rat DNA. There are two point mutations (or strain differences) in the Sprague-Dawley rat sequence at position 72 and 78, both of which are in the promoter sequence, not coding for any amino acids in BHMT. Intron 1 begins at position 143 ('GTGATG...').



**Figure 2.9** Alignment of experimentally determined Sprague-Dawley *BHMT* sequences and published Wistar rat genomic sequence. The experimentally determined sequences have three point mutations, or strain differences with the genomic Wistar rat sequence, at position 46, 72 and 78 above, but neither is in Exon1.

## 2.10 Discussion

The heat denaturation step was found to be simple and efficient, and provided a 5 fold purification in a single step, particularly when used as the initial step of BHMT purification. It was clear that prolonged exposure to high temperatures was efficient at denaturing the proteins in the crude liver homogenate, and that this denaturation occurs faster at higher temperatures. What was unique is the durability of BHMT to temperatures as high as 90 °C for as long as 20 min. Heat denatured protein was also easily separated from the active soluble BHMT.

It is unclear why BHMT that was eluted from the DEAE ion-exchange was unstable. One explanation is that there was an essential anionic cofactor or coenzyme which is removed under these conditions, although there are no known cofactors (other than zinc, which is cationic) for BHMT activity. The DEAE may contain some impurity which is inhibitory to BHMT activity, although the same loss of BHMT activity was observed after several washes of the column. This loss of activity was similar to the observations of Lee *et al*, who noticed their BHMT fractions were unstable unless stored with homocysteine and DMG.<sup>7</sup> It maybe that there is an inhibitor leaching from the DEAE gel, slowly affecting the stability of BHMT activity, or that there is an unidentified co-factor required for the stabilisation of mammalian liver BHMT.

The instability may also reflect the change to TRIS buffer, which is known to inhibit some enzymes.<sup>10-12</sup> TRIS (*tris*-(hydroxymethyl)aminomethane) contains a chemically active primary amine moiety, which is known to react in aqueous environments with common biological molecules.<sup>13</sup> It is possible that mammalian BHMT is similarly inhibited by TRIS buffer, although Garrow *et al* employ TRIS buffer in their preparations without similar problems in their recombinant BHMT solutions.<sup>14</sup>

Anion exchange chromatography was not used in the purification protocol for BHMT from liver tissue.

There has been controversy regarding the oligomeric state of BHMT. Some groups have reported that it is a hexameric protein,<sup>7,15</sup> others (particularly those relying on the crystal structures that have been obtained from recombinant BHMT) suggest a tetrameric protein.<sup>2,3</sup> The work of Waditee and Incharoensakdi on BHMT from *Aphanothece halophytica*, showed evidence that, at least in this species, (not mammalian) an octomeric protein was the active oligomeric state.<sup>16</sup>

The results obtained here suggest that the hexameric state is much more likely for purified rat liver BHMT. However, the measurement obtained is smaller than the expected mass for a hexamer by 29 kDa. There are at least two potential explanations for this result. First, while mass is the major factor in determining the elution volume of a protein, undergoing size exclusion chromatography, the protein shape also influences the result. Second, it has been hypothesised that BHMT-2 could form a heterodimer with BHMT.<sup>14</sup> If this was the case, then the resulting heterotetramer protein would have a lower mass (BHMT-2 subunits are predicted to have a lower mass than BHMT – 40.4 kDa vs 45.0 kDa, respectively, for human proteins). If this was occurring, say in a 4 x BHMT- 2 x BHMT2 configuration, then the predicted mass of the resulting hexamer would be 260.6 kDa (based on human proteins; the sequence for rat BHMT-2 is unavailable). This result is similar to the results obtained in the experiment, that would support this hypothesis, although no 40 kDa band was observed under SDS-PAGE analysis [Section 2.6].

The combination of heat denaturation and size exclusion chromatography, a similar purification to the procedures used by Garrow in 1996.<sup>6</sup> Garrow reports a final purification of 47 fold over the crude homogenate obtained from porcine liver.<sup>6</sup> The purification observed here was 33.1 fold. SDS-PAGE analysis [Figure 2.3] revealed a single dominant band at ~45 kDa size, which corresponded to the expected size of the BHMT subunit (44,970 Da). The

resulting protein was of an adequate homogeneity to proceed to kinetic characterisation, as established by SDS-PAGE analysis.

Approximately 75% of the BHMT subunit was mapped using the tryptic digest procedure. This information, and with the ~45 kDa protein band observed in SDS-PAGE analysis of the BHMT active fractions and the *N*-terminal analysis seen in **Section 6.4**, allow the positive identification of BHMT in the rat liver extract.

There was one possible N-linked glycosylation site, however the tryptic peptide containing this site was clearly identified in the mass spectrum, indicating that there was no glycosylation at this site.



## 2.11 Conclusion

The purification of BHMT from rat liver was effected simply using procedures which are commonly employed in protein purification. Heat denaturation was employed as an initial purification step, providing 7-fold purification over the crude homogenate. Size exclusion chromatography of the supernatant after heat shock gave a 33-fold purification over the crude extract, which is comparable to the purification factors quoted by other groups.<sup>6,7</sup>

The mass spectral data obtained also indicated the presence of BHMT subunits in the solution, although no data could be obtained on the BHMT unit as a whole. The possibility exists that there was post-translational modification of the BHMT subunit, but the data obtained were unable to identify any. However, the work done with the tryptic digest does suggest several locations where those modifications are unlikely. The fact that those fragments were observed, with the predicted mass, indicates that it is not likely that there is modification there.

## References

- (1) Breksa, A. P., III; Garrow, T. A. *Biochemistry* **1999**, Recombinant human liver betaine-homocysteine S-methyltransferase: Identification of three cysteine residues critical for zinc binding., *38*, 13991-13998.
- (2) Gonzalez, B.; Pajares, M. A.; Martinez-Ripoll, M.; Blundell, T. L.; Sanz-Aparicio, J. *Journal of Molecular Biology* **2004**, Crystal Structure of Rat Liver Betaine Homocysteine S-Methyltransferase Reveals New Oligomerization Features and Conformational Changes Upon Substrate Binding, *338*, 771-782.
- (3) Evans, J. C.; Huddler, D. P.; Jiracek, J.; Castro, C.; Millian, N. S.; Garrow, T. A.; Ludwig, M. L. *Structure* **2002**, Betaine-Homocysteine Methyltransferase Zinc in a Distorted Barrel, *10*, 1159-1171.
- (4) Millian, N. S.; Garrow, T. A. *Archives of Biochemistry and Biophysics* **1998**, Human betaine-homocysteine methyltransferase is a zinc metalloenzyme, *356*, 93-98.
- (5) Durell, J.; Anderson, D. G.; Cantoni, G. L. *Biochimica et Biophysica Acta* **1957**, Synthesis of methionine by enzymic transmethylation. I. Purification and properties of thetin homocysteine methylpherase, *26*, 270-282.
- (6) Garrow, T. A. *Journal of Biological Chemistry* **1996**, Purification, kinetic properties, and cDNA cloning of mammalian betaine-homocysteine methyltransferase, *271*, 22831-22838.
- (7) Lee, K. H.; Cava, M.; Amiri, P.; Ottoboni, T.; Lindquist, R. N. *Archives of Biochemistry and Biophysics* **1992**, Betaine:homocysteine methyltransferase from rat liver: purification and inhibition by a boronic acid substrate analog, *292*, 77-86.
- (8) Ericson, L. E. *Acta Chemica Scandinavica* **1960**, Betaine-homocysteine methyltransferases. II. Isolation and properties of the transferase of pig liver, *14*, 2113-2126.
- (9) Finkelstein, J. D.; Harris, B. J.; Kyle, W. E. *Archives of Biochemistry and Biophysics* **1972**, Methionine metabolism in mammals: kinetic study of betaine-homocysteine methyltransferase., *153*, 320-324.
- (10) Betts, G. F.; Evans, H. J. *Biochimica et Biophysica Acta* **1968**, Inhibition of univalent cation-activated enzymes by tris(hydroxymethyl)aminomethane, *167*, 193-196.
- (11) Kano, T.; Usami, Y.; Adachi, T.; Tatematsu, M.; Hirano, K. *Biological & Pharmaceutical Bulletin* **1996**, Inhibition of purified human sucrase and isomaltase by ethanolamine derivatives, *19*, 341-344.

- (12) Sanker, S.; Sivakami, S. *Journal of Biosciences (Bangalore, India)* **1988**, Purification and properties of trehalase from monkey small intestine, *13*, 153-158.
- (13) Bubbs, W. A.; Berthon, H. A.; Kuchel, P. W. *Bioorganic Chemistry* **1995**, Tris buffer reactivity with low-molecular-weight aldehydes: NMR characterization of the reactions of glyceraldehyde 3-phosphate, *23*, 119-130.
- (14) Garrow, T. *Personal Communication*.
- (15) Skiba, W. E.; Taylor, M. P.; Wells, M. S.; Mangum, J. H.; Awad, W. M., Jr. *Journal of Biological Chemistry* **1982**, Human hepatic methionine biosynthesis. Purification and characterization of betaine:homocysteine S-methyltransferase, *257*, 14944-14948.
- (16) Waditee, R.; Incharoensakdi, A. *Current Microbiology* **2001**, Purification and kinetic properties of betaine-homocysteine methyltransferase from *Aphanothece halophytica*, *43*, 107-111.

## Chapter 3

# NMR Spectroscopic Assays

## CHAPTER 3: NMR Spectroscopic Assays

### 3.1 NMR Concepts

#### 3.1.1 Introduction

Current assay techniques for BHMT activity are all unsuitable for high throughput analysis of multiple substrates or for rapid assessment of kinetic results. Glycine betaine does not contain a chromophore, and is not fluorescent. What is more, DMG and methionine are also limited as indices of BHMT activity, since both compounds are also difficult to quantify.

There is interest in the effects of betaines on BHMT activity, because these may be used as methyl group donors to lower homocysteine in people with heart or kidney disease. This has been recently called into question, following the reports on the NORVIT (Norwegian Vitamin) study,<sup>1</sup> and the HOPE (Heart Outcomes Prevention Evaluation) study,<sup>2</sup> both of which found that folate and vitamins B<sub>6</sub> and B<sub>12</sub> supplementation in CVD patients lowered plasma homocysteine, but was not associated with a lower risk of further cardiovascular events. These studies have been used to claim that the 'Homocysteine Hypothesis is dead', that is that there is no causal link between homocysteine and CVD.<sup>3</sup> Neither study included betaine supplementation in their regimes, which have been shown to be effective in lowering homocysteine.<sup>4-9</sup> Despite some interpretations of the NORVIT and HOPE studies, the homocysteine hypothesis has not been disproved by their research.<sup>3</sup> These two studies do not remove the overwhelming evidence that homocysteine is an atherogenic determinant.<sup>10</sup> The possibility that excessive folate and vitamin B<sub>12</sub> supplementation may increase the risk of CVD through hypermethylation has not been discussed or eliminated.<sup>11,12</sup> The possibility remains

that BHMT, using glycine betaine as a methyl donor, is a much more important regulator of plasma homocysteine. For this reason, research into BHMT metabolism and regulation should be pursued, and analogues of glycine betaine which may assist in lowering homocysteine through BHMT should be sought.

### 3.1.2 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) has rapidly become one of the most powerful techniques in chemical analysis since its inception in 1945.<sup>13</sup> It has become such a powerful technique, that multiple Nobel prizes have been awarded to people in the field of NMR, including F. Bloch and E.M. Purcell, who won the Nobel Prize in Physics in 1954 for their work on NMR, and for the first experimental evidence of the NMR effect. R.R. Ernst earned the Nobel Prize in Chemistry in 1991 for developing the 2-dimensional technique.<sup>14</sup>

It is one of the few truly non-destructive analytical techniques that exists in chemistry, and has found wide application in fields as diverse as solid-state engineering, surface chemistry, protein structure analysis, medical imaging and other biological analyses.<sup>15</sup>

### 3.1.3 Spin, and the Nuclear Magnetic Resonance Effect

NMR is the result of a quantum effect called 'nuclear spin'. Nuclear spin arises from the subatomic particles making up the nucleus, which also have a characteristic 'spin' number associated with them. This spin effect is attributable to all subatomic particles, however, some have an overall spin of zero.

This thesis will not probe the exact nature of quantum spin or its causes, nor will it outline in any detail the workings of the NMR spectrometer. However, a brief analysis of spin

and the NMR effect will enable a greater understanding of the information presented later in the chapter.

Total nuclear spin can take only integer or half integer values (quanta) which are always multiples of  $I = \frac{1}{2}$ . Nuclei with spin of zero ( $I = 0$ ) do not exhibit an NMR resonance and so do not feature in any NMR experiment. Most elements contain at least one isotope with  $I > 0$ , enabling analysis by NMR spectroscopy. The most abundant nucleus in the universe, and in most organic/biological chemistry, is the hydrogen nucleus ( $^1\text{H}$ ), with  $I = \frac{1}{2}$ . Of note is also  $^{13}\text{C}$ , which gives an NMR signal ( $I = \frac{1}{2}$ ) but is only present at 1.1% natural abundance, and so measurements of it are not as sensitive as  $^1\text{H}$ -NMR spectroscopy.

Possessing a nuclear spin in effect gives the nucleus an angular momentum vector, which, combined with the charge in the nucleus, gives them a weak magnetic field ( $\mu$  – the nuclear magnetic dipole moment), enabling them to respond to external magnetic fields in the same manner as macroscopic magnets do. Moreover, the nucleus will precess with a frequency known as the Lamor frequency.

For any given proton, the nuclear magnetic dipole moment ( $\mu$ ) is given by **Equation 3.1**, where  $m_p$  is the mass of the proton,  $e$  is the charge on one electron and  $\hbar$  is the Planck constant:

$$\mu = \frac{e\hbar}{2m_p} = 5.051 \times 10^{-27} \text{ JT}^{-1} \quad \text{Equation 3.1}$$

In the presence of a large external magnetic field ( $\vec{B}$ ),  $\mu$  orients in accordance, and for a nucleus with spin =  $I$ , there are  $2I + 1$  possible orientations (for a proton,  $I = \frac{1}{2}$ , and the number of orientations is 2,  $\pm\frac{1}{2}$ ). The energies of these orientations ( $m_I$ ) are given by **Equation 3.2**:

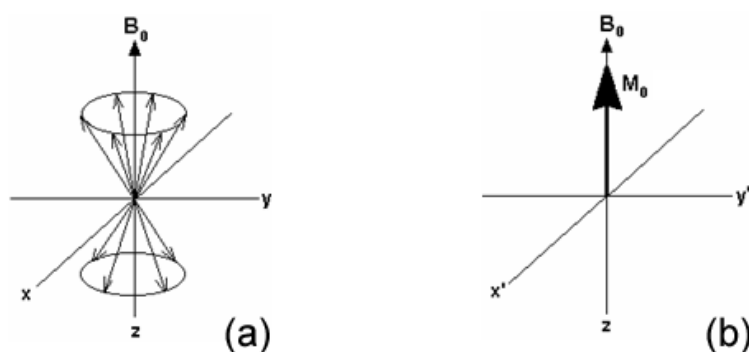
$$E_m = -g_I\mu_I\vec{B}m_I \quad \text{Equation 3.2}$$

Where  $g$  is the nuclear  $g$ -factor: an experimentally determined characteristic of the nucleus. If  $g$  is a positive number, then the nucleus is aligned with the external field, if  $g$  is negative, then the nucleus is aligned against the field. In the presence of a large external magnetic field, two energy levels are formed, and there is an energy gap given by the difference of these two levels:

$$\Delta E = g_1 \mu_1 \bar{B} \quad \text{Equation 3.3}$$

If a photon can be introduced with energy that corresponds to the energy gap, as determined by the external magnetic field, then the nucleus can be induced to 'flip' from spin up to spin down. This gives rise to the resonance effect.

In any given sample, however, there are a large number of nuclei present, and given the same chemical environment, they will resonate with the same frequency [Figure 3.1 (a)]. Hence, individual  $\mu$  vectors can be combined into a larger summed vector, named the 'bulk magnetisation vector', ' $M$ ' which is further described in the diagram Figure 3.1 (b).

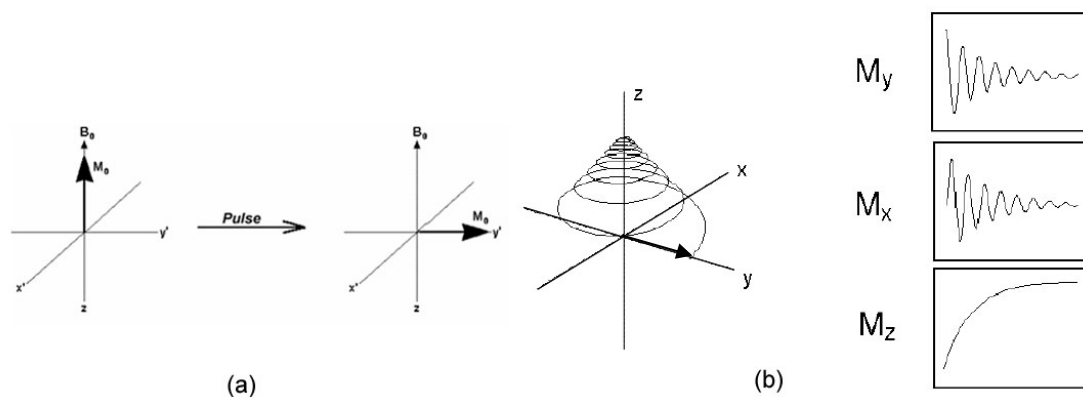


**Figure 3.1** The combination of individual  $\mu$ 's (a) to give a bulk magnetization vector  $M$  (b).

The response produced and measured by the NMR spectrometer is a result of the bulk magnetization vector, and analysis of the pulse sequences can also be thought of as acting on the bulk magnetization vector. As RF pulses are applied to the sample in the spectrometer,  $M$  rotates along the axis of the RF pulse, changing the direction by an angle which depends on



the duration of the RF pulse. At the appropriate duration, this results in the magnetization lying in the  $x'-y'$  plane, known as *transverse magnetization*. As this magnetization returns to the ground state of  $M_0$ , it undergoes relaxation, that is, it loses the energy that was added to the system [Figure 3.2].



**Figure 3.2** Bulk magnetization vector after a  $90^\circ$  pulse (a), and the resulting relaxation process back to the  $Z'$ -axis (b). The  $M$  vector along each axis is shown.

There are two ways a nucleus can 'relax' – or 'lose' that energy back to the environment. The first is via *longitudinal relaxation*, the process by which the population of nuclei return to the ground energy state from which they came. This process gives rise to the spiral seen in **Figure 3.2 (b)**, and also is the process from which the NMR signal, or Free Induction Decay (FID) is produced. This relaxation occurs via an exponential function, with an exponential constant called  $T_1$ .

The second relaxation process, called *transverse relaxation*, arises from magnetic field inhomogeneity, the proximity of other  $I = \frac{1}{2}$  nuclei which can interact with the nucleus, and the molecular dynamics of the species in solution. This also fits an exponential decay function, and is given the constant  $T_2$ .

### 3.1.4 Pulse Sequences

Modern NMR spectrometers are capable of applying a number of RF signals ('pulses') to the samples during the course of NMR experiments. These normally exploit one of the parameters mentioned above to enhance or reduce a signal or set of signals which is problematic within a spectrum. There are several of these parameters which were used during the course of this thesis, and the pulse sequences are detailed within this chapter.

In Fourier Transform NMR (FT-NMR) spectroscopy, a pulse is applied in the form of monochromatic RF radiation at the Larmor frequency of the protons of interest. In practice, the *excitation pulse* (or *hard pulse*) is a short one, of the order of 10  $\mu\text{s}$ , so the Heisenberg uncertainty principle describes a considerable uncertainty on that frequency; effectively the pulse is a polychromatic one, covering the entire window of the spectrum. The duration of the pulse ( $\Delta t$ ) is referred to as the pulse width.

If a longer, weaker pulse is applied to a region of the spectrum (known as a *selective excitation*, or *soft pulse*) then the uncertainty on the frequency is lower, and the pulse excites a small part of the spectrum, selectively.

## 3.2 Quantitative NMR Spectroscopy<sup>16</sup>

### 3.2.1 Introduction

The NMR experiment as it is normally conducted is not a quantitative one. In principle, the integrals of peaks from the  $^1\text{H}$ -NMR experiment should reflect the number of nuclei which gave rise to them. That is, a  $-\text{CH}_3$ ,  $-\text{CH}_2$  and  $\text{CH}$  group should give rise to relative integrals of 3, 2, and 1, respectively, regardless of the species in the sample tube which gave rise to them. The integrals from a single pulsed Fourier Transform  $^1\text{H}$  NMR (FT-NMR) experiment are not normally quantitative, however, and can often have an error of 20-30%.

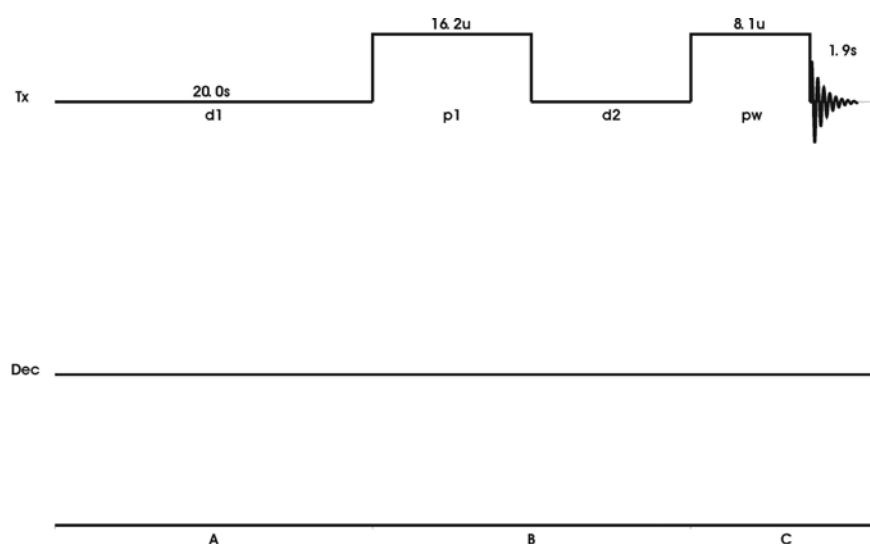
### 3.2.2 $T_1$ Relaxation

In order for quantitative measurements to be gathered from the NMR experiment, several considerations must be taken into account. First, if the nuclei are not allowed to relax fully to their ground state then the second  $90^\circ$  RF pulse will flip them past  $90^\circ$ . Consequently, their signals will decrease from each subsequent iteration of the pulse sequence. To avoid this, a delay is inserted between each iteration, enabling  $\mathbf{M}$  to fully return to the ground state magnetization. In practical terms, a value of  $5 T_1$  is used and is easily adopted in  $^1\text{H}$ -NMR spectroscopy.

Measuring  $T_1$  values is performed by a pulse sequence known as '*inversion recovery*'. Essentially, the sample is subjected to a  $180^\circ$  pulse, allowed to recover and then a further  $90^\circ$  pulse is followed by data acquisition [Figure 3.3]. The recovery delay is varied over a range of values, and as the sample relaxes, the bulk magnetisation vector returns along the  $z'$  axis to zero, and then back to the ground state. The rate at which this occurs is a direct measure of  $T_1$  (there is no net x-y magnetisation, so there is little influence from  $T_2$ ). The second  $90^\circ$  pulse

will take the bulk vector and place it along the  $y'$  axis, which then allows a signal which can be acquired and Fourier transformed to give a spectrum.

A plot of the final peak versus relaxation time will show an 'inverted' signal that passes through zero (when the bulk magnetization vector passes through the origin – no resulting signal along the  $y'$  axis) and finally into a positive signal. If the delay is sufficiently long enough, the  $180^\circ$  pulse will relax away so that it has no effect on the size of the spectrum. An example is shown in **Figure 3.4**.



**Figure 3.3** A typical 'inversion recovery' experiment used for measuring  $T_1$ . The 'p1' pulse is a hard  $180^\circ$  pulse which 'inverts' all the resonances along the  $z$  axis. The 'd2' delay is arrayed over a range of times (in s) to allow the signals to partially recover before 'pw', a  $90^\circ$  pulse, and data acquisition. 'D1' is set high (20 s) to allow total relaxation before the next iteration of the experiment.

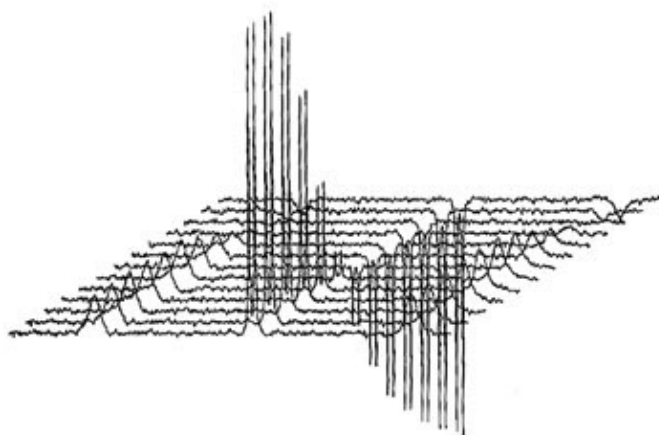
The time required to give a zero point spectrum ( $\tau_{\text{null}}$ ) can be used to gauge the value of  $T_1$ . This follows from the exponential nature of  $T_1$  relaxation:

$$M_t = M_0(1 - 2e^{-\tau/T_1}) \quad \text{Equation 3.4}$$

Where  $M_t$  is proportional to the magnitude of the signal observed after delay  $\tau$ .  $M_0$  is the initial magneton, seen at  $\tau = 0$ , and  $T_1$  is the exponential decay constant for longitudinal relaxation. It follows that:

$$T_1 = \frac{\tau_{\text{null}}}{\ln 2} = 1.443 \tau_{\text{null}}$$

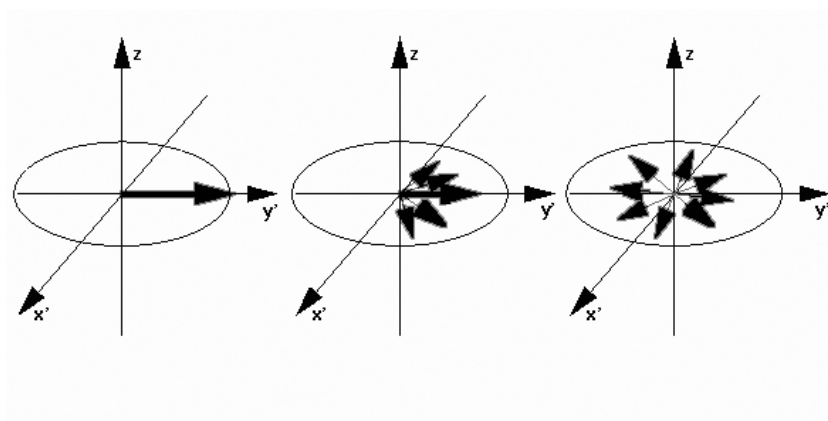
Equation 3.5



**Figure 3.4** An example of the result from an 'inversion recovery' experiment, as outlined in the text. The delay required to give a 'zero point' spectrum, where no resultant NMR signal is seen, is used to gauge the value of  $T_1$ .

### 3.2.3 $T_2$ Relaxation

There is a second mechanism by which nuclei can release the energy imparted by the pulse, known as transverse relaxation, or  $T_2$ . Where  $T_1$  was the loss of magnetisation along the  $z'$  axis, ultimately giving a null signal, transverse relaxation or  $T_2$  relaxation results in a 'blurring' or fanning of the individual magnetisation vectors in the  $x'$ - $y'$  plane [Figure 3.5].



**Figure 3.5**  $T_2$  relaxation. The bulk magnetisation vector along the  $y'$  axis is lost as individual groups of magnetisation vectors, often isochromats, with different Larmor frequencies.

In terms of observable effects on the acquired spectrum, having a rapid relaxation from  $T_2$  effects (a 'short'  $T_2$ ) will lead to line broadening in the post-Fourier transform spectrum.

The field inhomogeneities that give rise to  $T_2$  relaxation can arise from two distinct but inseparable sources. The first is static field inhomogeneity, i.e., the magnetic field is not the same over the sample volume; some nuclei experience a larger field than others, and so the Larmor frequency is different for these nuclei. Correctly calibrating or 'shimming' the NMR magnet prior to spectrum acquisition minimises effects resulting from an irregular magnetic field.

The second major contributing factor giving rise to transverse relaxation is small local differences in magnetic field resulting from molecular components in solution – i.e., arising from solution components.

### 3.2.4 Internal Standards for Quantification

Another consideration for quantitative NMR spectroscopy is that there is no equivalent to the *molar extinction coefficient* ( $\xi$ ) from UV spectroscopy. That is, the peak integrals cannot easily be converted to absolute concentrations from the standard FT-NMR experiment. In order

to quantify the concentration of substances present, an internal standard of known concentration must be present to give a measure of the relative intensity to the sample.

Internal standards must be carefully chosen. It must be non-reactive (in order to not interfere with the chemicals present in the sample) and ideally give rise to a singlet which is easily discernable from the rest of the spectrum. For kinetic analysis, there is the extra requirement for the reference compound to be kinetically neutral to the enzyme. It was found that *tert*-butanol did not affect BHMT activity, so was used for kinetic measurements of BHMT [Chapters 4, 5, 6]. Acetonitrile is not a normal component of mammalian urine, so was used for quantitative measurements in urine [Chapter 7].

### 3.2.5 Aqueous Measurements

Water presents a problem for  $^1\text{H}$ -NMR spectroscopic measurements. Since the water molecule contains two protons, and is present at 55 M concentrations, it gives rise to a dominating peak (110 M  $^1\text{H}$  equivalent) at about 4.7 ppm. Furthermore, the protons on the water peak are in rapid exchange with any amine or hydroxyl peaks in the sample.

There are several methods for removal of water signals from  $^1\text{H}$ -NMR spectra. The simplest is the presaturation technique, which uses a selective irradiation on the water signal for several s prior to the  $90^\circ$  pulse, which effectively scrambles the magnetisation of water protons, giving them no signal, therefore removing them from the spectrum. The consequences of this technique include the substitution of the excited protons from the solvent to exchangeable protons, like that on the hydroxy, or amine moiety. However, there is little disturbance to the baseline when this technique is used. A second technique used during the course of the work of this thesis was the double pulsed field gradient spin echo (DPFGSE) technique. This technique uses field gradients to selectively excite the solvent molecules, scrambling their magnetisation and therefore rendering them invisible to the proton spectrum.

The consequences of this technique include the distortion of the baseline in ways which can be obstructive to the quantification of the peaks in the spectrum, particularly if the underlying molecules differ significantly in concentration.<sup>17</sup> The DPGSE, however, achieves much better suppression of the solvent peaks than the presaturation technique. There are other techniques which were not employed in the course of this thesis, including WATR (Water Attenuation by Transverse Relaxation).<sup>18-20</sup>

### 3.3 Relaxation Measurements of Methylamines

#### 3.3.1 Introduction

In order to set appropriate delays in the pulse sequences being used in NMR spectroscopy, the  $T_1$  relaxation measurements must be made. There are standard procedures for the measurement of  $T_1$ , which have been pre-loaded into the Varian software (used for this work), and are evoked with the command 'dot1'. This automatically initiates a 180° - delay - 90° - acquire pulse sequence (as described in **Section 3.2.2**) with incrementally increasing delays. This sequence, however, provides no water suppression and so the 'inversion-recovery' sequence must be modified with some form of presaturation between iterations. Fortunately, a 3 s presaturation pulse can be introduced before the inversion recovery. Unfortunately, once significant delays are employed in the pulse sequence (i.e., in the 3-4 s range) the effects of the presaturation pulse begin to wane, and can swamp any nearby peaks that are being analysed.

#### 3.3.2 Method

Aqueous solutions of glycine betaine, dimethyl glycine, methionine, acetonitrile and *tert*-Butanol were prepared at 5 mM and 100  $\mu$ M. The pulse sequence described above



(inversion recovery, with presaturation on the water resonance for three s) was introduced to the sample and d1 was arrayed over the values 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 s.

### 3.3.3 Results

Each of the small metabolites extinguished during the 1.6 s array experiment, indicating that the  $T_1$  decay constant was  $\sim 1.6/\ln 2 = 2.3$  s. This measurement remained constant over concentrations, with the 5 mM and the 100  $\mu$ M sample giving the same 2.3 s  $T_1$  value.

## 3.4 Validation of $^1\text{H}$ -NMR Spectroscopy for Urine Methylamine Measurements

### 3.4.1 Introduction

Alternative methods for measuring methylamine metabolites in urine were investigated in response to two clinical demands. First, there has been increasing recent interest in betaines and betaine metabolites because of their association with homocysteine metabolism<sup>8,21-24</sup> and, hence, with vascular disease.<sup>8,25</sup> Secondly, trimethylamine (TMA) excretion is increased in patients suffering from a genetic disorder known as 'fish odour syndrome'.<sup>26,27</sup> An increase in the ratio of TMA to trimethylamine *N*-oxide (TMAO) is diagnostic for this condition, and useful for diagnosing heterozygotes. While this is not a fatal condition, it inflicts a characteristic odour which can be socially debilitating.

In previous work, the Osmolyte Research group at Canterbury Health Laboratories, in Christchurch, have used the HPLC separation of aracyl derivatives to measure these metabolites.<sup>25,28-31</sup> This is satisfactory for plasma but with urine samples there are a large number of peaks, and to get adequate resolution it is necessary to resort to long run-times (>60 min). Even so, it is difficult to resolve all the metabolites of interest in one chromatogram. Additionally, TMA and TMAO give the same derivative and are not separately

quantified. In an animal study<sup>23</sup> it was found that <sup>1</sup>H-NMR spectroscopy could be used to measure all the metabolites of interest rapidly and at the same time, and subsequent experience<sup>32</sup> suggested that this technique could be used to provide a clinical diagnostic service.

NMR spectroscopy has been used for the identification and quantification of a number of metabolites in urine and plasma. In the early 1980's, <sup>1</sup>H-NMR spectroscopy established itself as a versatile tool for the analysis of whole urine. The early work of Bales *et al*, and Nicholson *et al* showed that useful clinical information could be derived from whole urine NMR work.<sup>33,34</sup> More recently, sophisticated techniques have been developed to suppress the water and protein signals which complicated those early spectra.<sup>35</sup> The use of NMR spectroscopy in clinical applications is becoming more commonplace, given its unique ability to identify and quantify multiple metabolites in a single run. Wide ranging clinical studies using NMR spectroscopy have been undertaken, including disturbed metabolism of TMA,<sup>27,36</sup> supplementation in athletes,<sup>37</sup> determination of novel metabolic disorders<sup>38</sup> and observation of renal clearance.<sup>39</sup> Urine betaine and TMAO analysis by <sup>1</sup>H-NMR spectroscopy has previously been problematic since they can easily be confused.<sup>40</sup> Experiments were undertaken to demonstrate that, for the group of methylamine metabolites studied, these problems can be resolved and a rapid and versatile diagnostic assay developed.

### 3.4.2 Method

#### 3.4.2.1 Materials

Glycine betaine, trigonelline, dimethylglycine (DMG), trimethylamine, trimethylamine *N*-oxide, acetonitrile, deuterium oxide and HCl were purchased from Sigma-Aldrich (St Louis, MO, USA). Proline betaine was synthesised using the method of Cornforth and Henry.<sup>41</sup>

#### 3.4.2.2 Sample Preparation

400  $\mu\text{L}$  of urine was added to 100  $\mu\text{L}$  of 1 M HCl solution containing acetonitrile at 25 mM as an internal standard.

#### 3.4.2.3 NMR Assay

All  $^1\text{H}$  NMR spectra were recorded on a Varian INOVA 500 instrument, at 23  $^{\circ}\text{C}$  in 5 mm NMR tubes with a 3 mm  $\text{D}_2\text{O}$  lock insert. For the measurements, a  $90^{\circ}$  radiofrequency pulse, with duration of 8.1  $\mu\text{s}$  was used. The delay between pulses was 5 s, acquisition time was 1.982 s. Eight transients were recorded for each sample, with 30,272 data points for each transient. Spectral width was set to 8,000 Hz. Water suppression was achieved using a presaturating irradiation of the water spin population of 3 s during the relaxation delay period. All spectra were zero filled to 128k points, and then Fourier transformed. Phasing and baseline corrections were completed manually. The software used for these procedures was VNMR version 6.1C (Varian), which also provides a signal-to-noise calculation facility for the determination of the limits of detection.

Quantification of the metabolites was performed using an internal standard of acetonitrile (5 mM). Peak integrals and peak heights were measured after baseline and drift correction and then expressed as a ratio to the acetonitrile integral.

Trigonelline was subject to the same analysis as the other betaines, but its proximity to the suppressed water resonance led to baseline distortions. Peak integrals proved to be unreliable for trigonelline, and a more reproducible measure was obtained from the peak height ratio to acetonitrile in a method similar to Fulton *et al.*<sup>42</sup>

#### *3.4.2.4 Precision and Recovery Study*

To make urine samples with elevated levels of betaines, 100  $\mu\text{L}$  of aqueous standards of the betaines was added (spiked) into 10 mL of urine. This resulted in a urine samples with elevated concentrations of the methylamines in question (spikes were: 100  $\mu\text{M}$  trigonelline, 750  $\mu\text{M}$  TMAO, 75  $\mu\text{M}$  glycine betaine and TMA, 140  $\mu\text{M}$  DMG and 2.5 mM creatinine). The unspiked urine sample with low betaine levels was also used in this precision study. Six batches with four replicates of the spiked and non-spiked urine were analysed.

#### *3.4.2.5 Linearity*

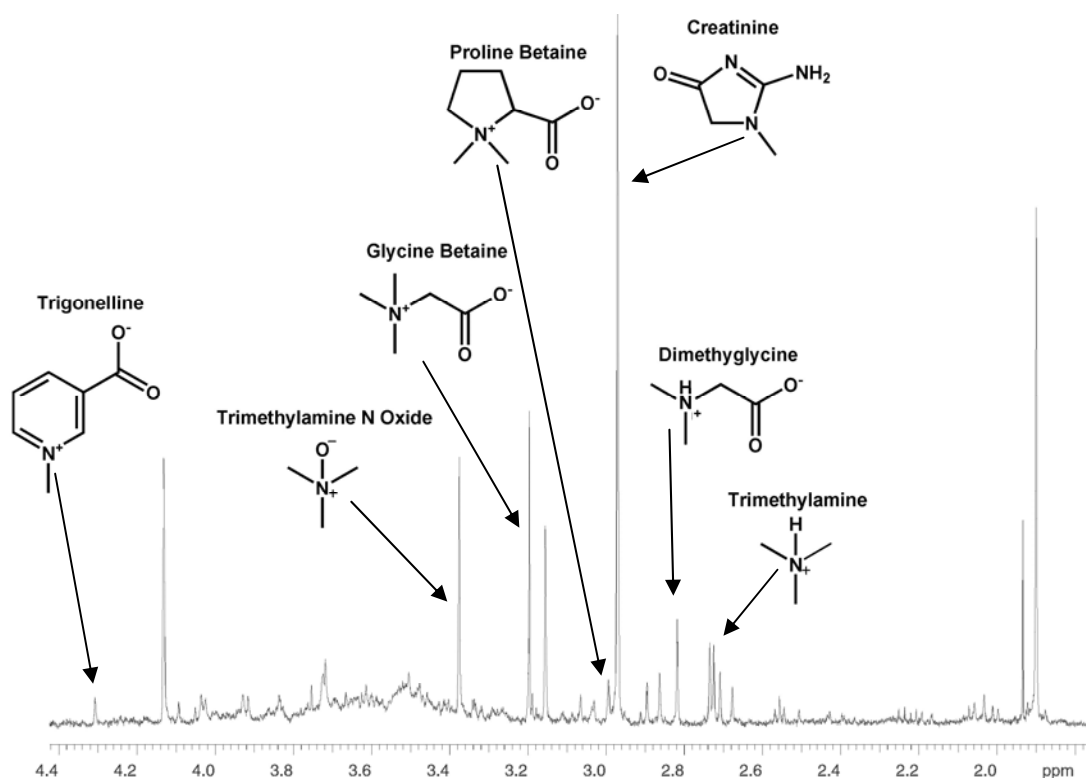
Standard solutions, at concentrations of 50 - 700  $\mu\text{M}$ , were prepared for each of trigonelline, glycine betaine, proline betaine and DMG. Because of differences in concentrations in samples, TMAO was prepared at 100 – 7000  $\mu\text{M}$ , TMA at 10 – 700  $\mu\text{M}$  and creatinine at 0.25 - 17.5 mM. These were prepared by serial dilution of a 10 mM aqueous stock standard. Samples were analysed and the linearity of the method was determined using linear regression analysis over six data points.

### **3.4.3 Results**

#### *3.4.3.1 Internal Standards*

Acetonitrile was used as an internal standard because its resonance is well separated from the resonances of the compounds of interest. The resonance positions of many metabolites are dependent on the solution pH, so the use of HCl was required to ensure that the pH of the samples was consistent between analyses. At low pH, the TMA peak was split into a doublet with coupling observed to the ammonium proton,<sup>43</sup> however, the methyl resonances were sufficiently removed from other metabolites for integration of both peaks to

be achieved. Lowering the pH to  $\sim 1$  was required for separation of TMAO and glycine betaine. A typical spiked spectrum is shown in **Figure 3.6**. Additionally, carnitine was observed at 3.07 ppm, and it did not interfere with any of the species that were measured.



**Figure 3.6** Spiked (300  $\mu\text{M}$ ) urine with methyl resonances for the metabolites studied.

#### 3.4.3.2 Spectrum Analysis

Baseline distortions, which were introduced after the water suppression was applied, were problematic for the methyl resonance of trigonelline, which was near the residual water resonance. The use of peak height data, which can be extracted after smoothing the baseline, was far more reliable than peak integral data when applied to trigonelline.<sup>42</sup>

*3.4.3.3 Precision*

The coefficient of variation (CV) was below 10% for within batch and between batch variation when using the internal standard calibrated data [Table 3.1]. A brief comparison of peak height and peak integral data was performed. The peak integral data were used for most of the metabolites presented here, because the baseline was flat and the peaks were well resolved. In the case of trigonelline, the residual water resonance caused a significant baseline distortion. Peak height proved to be more reliable, with artificial baseline correction performed using the Varian VNMR software. Peak heights were difficult to obtain for the other metabolites, as the neighbouring signals made the same baseline corrections complicated.

**Table 3.1** Results of precision and recovery study of betaines in urine with betaine spike and unspiked sample (four batches, n=6). (\* Trigonelline results obtained from peak height data.)

Analyte		Mean μM	Within batch CV	Between batch CV	Recoveries %
Trigonelline*	low urine	75	4.8	4.1	98
	spike	178	6.9	2.3	
TMAO	low urine	976	4.3	2.0	105
	spike	1760	2.5	8.5	
Proline betaine	low urine	67.0	4.7	2.3	98
	spike	165	2.9	2.8	
Glycine betaine	low urine	63.1	5.8	6.2	100
	spike	138	4.5	3.0	
Dimethylglycine	low urine	60.5	6.0	5.7	98
	spike	197	4.1	3.1	
TMA	low urine	8.4	7.4	6.6	100
	spike	83	4.2	5.1	
Creatinine	low urine	3950	0.9	0.7	97
	spike	6370	0.6	3.0	

*3.4.3.4 Limits of Detection*

Limits of detection (S/N =3) for each compound are shown in Table 3.2. The precision of this NMR method was good at both low levels (unspiked sample) and high levels (100 μM).

## 3.4.3.5 Accuracy

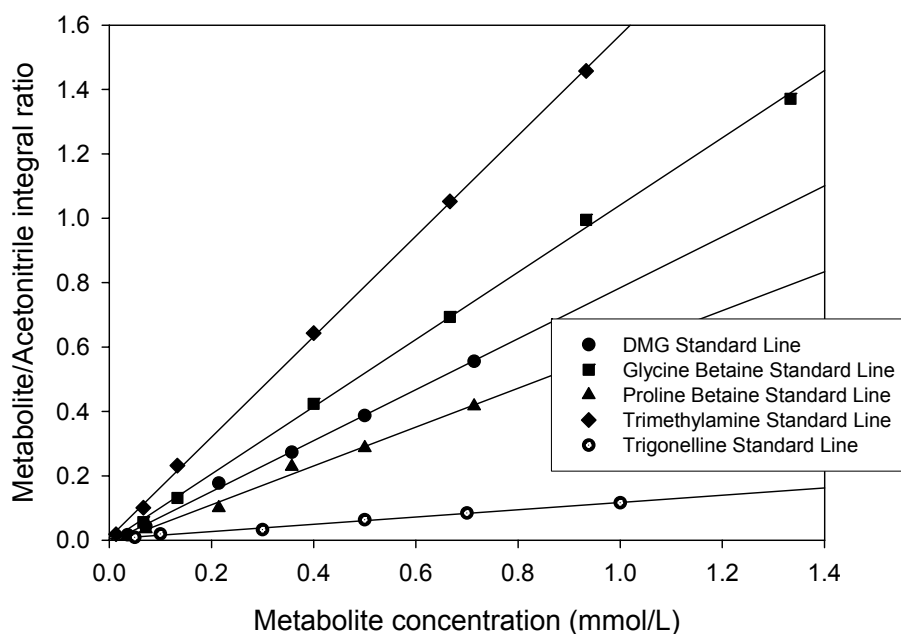
Recoveries of betaines were over 95% for all betaines in urine. The recovery values given in **Table 3.1** show that all of the betaines of interest could be measured accurately.

**Table 3.2** Chemical shift (relative to acetonitrile at 1.9 ppm) and limit of detection (LOD) for the metabolites analysed. (\* Trigonelline results obtained from peak-height data.)

Analyte	Chemical Shift (ppm)	LOD ( $\mu\text{M}$ )
Trigonelline*	4.30	25
TMAO	3.37	15
Proline betaine	2.99	25
Glycine betaine	3.19	15
DMG	2.82	20
TMA	2.73, 2.72	15
Creatinine	2.97	25

## 3.4.3.6 Linearity

The NMR assay was linear over the range of 50  $\mu\text{M}$  - 1000  $\mu\text{M}$  for trigonelline, glycine betaine, proline betaine and DMG, 100 – 7000  $\mu\text{M}$  for TMAO, 10 – 700  $\mu\text{M}$  for TMA and 0.250 - 17.5 mM for creatinine. Standard lines for these metabolites are shown in **Figure 3.7**.



**Figure 3.7** Standard lines for the metabolites measured by  $^1\text{H}$ -NMR Spectroscopy. TMAO and creatinine were omitted because of differences in scale, however, all methylamines were linear over the specified ranges.

#### 3.4.3.7 TMA Analysis

Two patients with suspected cases of trimethylaminuria were given a load of 600 g of fish and had a urine sample taken 12 h later. Their TMA and TMAO concentrations were determined and the percentage ratio of TMA and TMAO are given at about 5% for both people. As a comparison, a normal control has about 1% ratio of TMA [Table 3.3].

**Table 3.3** TMA and TMAO measurements of two suspected cases of heterozygous trimethylaminuria and a normal control.

	TMA	TMAO	Ratio TMA/TMAO	% TMA/ TMA+TMAO	%TMAO/ TMA+TMAO
Patient 1	30.01	603.8	0.05	4.7	95
Patient 2	56.22	1102.27	0.04	4.8	95
Control	2.09	236.05	0.009	0.9	99



### 3.5 Discussion

The results of the first, inversion-recovery experiment placed limits on the delays set into the NMR experiment. In reality, the need for consistent delays between calibration and sample allows for the relaxation of the samples, but delays should be well over  $4 \times 2.3$  s to ensure complete relaxation. The parameters developed in the inversion-recovery experiment were used in the validation of the NMR assay for clinical applications.

Measurement of methylamine metabolites by  $^1\text{H}$ -NMR spectroscopy is a rapid, simple, non-destructive and effective quantitative method, involving minimal sample preparation and the capacity to analyse many samples in a short time (2-3 minutes per sample). As well as the metabolites presented here, the method could be adapted to measure other methylated substances, for example carnitine (which does not interfere with the urine components that were the subject of the present studies). Moreover, when an unknown metabolite occurs in a urine sample, the NMR spectrum can provide substantial information about its nature. If required, 2D NMR spectroscopy can also be run on the sample with no further preparation and structural information gathered.

Previously, other groups have used NMR spectroscopy for measuring these methylamines. Lundberg *et al* quantified DMG and glycine betaine in the urine of patients with premature vascular disease.<sup>44</sup> A new inborn error of metabolism giving elevated urine excretion of DMG was identified similarly,<sup>38</sup> and we used this technique to observe changes in DMG excretion in a DMG supplementation study.<sup>45</sup> Often water is evaporated off and replaced with deuterium oxide,<sup>27</sup> however, it is shown here that the water signal can be suppressed without this time-consuming step. Alternative methods that have been used for water suppression in  $^1\text{H}$ -NMR spectroscopy include Double Pulsed Field Gradient Spin Echo (DPFGSE), WATERGATE and WET pulse sequences.<sup>23,35</sup> Presaturation was chosen here, because none of the signals in question were in rapid exchange with water. It is essential to lower the pH of

the urine to pH~1 to correctly assign the signals. Previously the resonance caused by TMAO ( $pK_a = 4.65$ ) in the urine of renal failure patients has been incorrectly attributed to glycine betaine ( $pK_a = 1.83$ ).<sup>40</sup> These are resolved after acidifying the samples, and this is necessary since both are likely to be present in substantial amounts in normal urine.

The methods described here are being used for studies of urinary betaines and vascular disease,<sup>25,32</sup> which require simultaneous measurement of glycine betaine and its metabolite DMG, along with the potentially confounding dietary betaines,<sup>46,47</sup> proline betaine and trigonelline. It is also being used for the diagnosis of TMAuria and an example of results with presumed heterozygous subjects is illustrated [Table 3.3]. However, the approach is far more general, for example the presence of clearly resolved carnitine peaks in normal urine samples suggests that carnitine and acylcarnitines could also be measured. Most groups have measured methylamine metabolites by combinations of chromatographic procedures with UV, fluorescence or mass-spectrometric detection: <sup>1</sup>H-NMR spectroscopy is a rapid non-destructive alternative.

### 3.6 Conclusion

The use of  $^1\text{H}$ -NMR spectroscopy for the measurement of methylamines is not new. Several groups have previously worked on the use of  $^1\text{H}$ -NMR spectroscopy to measure chemicals in biofluids, including urine, plasma, and cerebro-spinal fluid.<sup>48-66</sup> These groups have measured various markers of disease, from trimethylamine (and its oxide) as a marker of fish-odour syndrome, to glucose for diabetes, to creatine excretion for doping studies (in France, where the substance is a banned athletic supplement). The application of  $^1\text{H}$ -NMR spectroscopy, to routine clinical measurements is only limited by the financial investment that the magnets require.

NMR spectroscopy is a superior method of analysis of methylamines to the currently available methods. Methods currently employed for methylamine analysis include HPLC and head-space gas chromatography - mass spectrometry (GCMS). Methylamines tend to lack chromophores, and are not generally fluorescent, so are not easily detected using HPLC. Furthermore, the analysis times for HPLC are up to 60 min, making high-throughput analysis difficult. GCMS of the headspace of a sample is only applicable to the analysis of trimethylamine and its oxide, and is often complicated by interfering compounds in the sample.

The studies presented here allow for the rapid detection and quantification of methylamine compounds. Furthermore, the techniques described should be applicable to most other compounds present in the urine, which may also be used as markers of disease. In most cases, quantitative measurements were completed within 5 min, and the sample preparations were far simpler than the chemical manipulations necessary for HPLC analysis. The CV's presented for the methylamines in these studies are comparable to those obtained in clinical assays, making NMR spectroscopy an attractive alternative to the techniques available now.

## References

- (1) Bonaa, K. H.; Njolstad, I.; Ueland, P. M.; Schirmer, H.; Tverdal, A.; Steigen, T.; Wang, H.; Nordrehaug, J. E.; Arnesen, E.; Rasmussen, K. *New England Journal of Medicine* **2006**, Homocysteine lowering and cardiovascular events after acute myocardial infarction, *354*, 1578-1588.
- (2) Lonn, E.; Yusuf, S.; Arnold, J.; Sheridan, P.; Pogue, J.; Micks, M.; McQueen, M. J.; Probstfield, J.; Fodor, G.; Held, C.; Genest, J., Jr. *New England Journal of Medicine* **2006**, Homocysteine lowering with folic acid and B vitamins in vascular disease, *354*, 1567-1577.
- (3) Loscalzo, J. *New England Journal of Medicine* **2006**, Homocysteine trials - Clear outcomes for complex reasons, *354*, 1629-1632.
- (4) Steenge, G. R.; Verhoef, P.; Katan, M. B. *Journal of Nutrition* **2003**, Betaine supplementation lowers plasma homocysteine in healthy men and women, *133*, 1291-1295.
- (5) Olthof, M. R.; van Vliet, T.; Boelsma, E.; Verhoef, P. *Journal of Nutrition* **2003**, Low dose betaine supplementation leads to immediate and long term lowering of plasma homocysteine in healthy men and women, *133*, 4135-4138.
- (6) Schwab, U.; Torronen, A.; Toppinen, L.; Alfthan, G.; Saarinen, M.; Aro, A.; Uusitupa, M. *American Journal of Clinical Nutrition* **2002**, Betaine supplementation decreases plasma homocysteine concentrations but does not affect body weight, body composition, or resting energy expenditure in human subjects, *76*, 961-967.
- (7) McGregor, D. O.; Dellow, W. J.; Robson, R. A.; Lever, M.; George, P. M.; Chambers, S. T. *Kidney International* **2002**, Betaine supplementation decreases post-methionine hyperhomocysteinemia in chronic renal failure, *61*, 1040-1046.
- (8) Olthof, M. R.; Verhoef, P. *Current Drug Metabolism* **2005**, Effects of betaine intake on plasma homocysteine concentrations and consequences for health, *6*, 15-22.
- (9) Craig, S. A. S. *American Journal of Clinical Nutrition* **2004**, Betaine in human nutrition, *80*, 539-549.
- (10) den Heijer, M. *Clinical Chemistry and Laboratory Medicine* **2003**, Hyperhomocysteinemia as a risk factor for venous thrombosis: An update of the current evidence, *41*, 1404-1407.

- (11) Ulrey, C. L.; Liu, L.; Andrews, L. G.; Tollefsbol, T. O. *Human Molecular Genetics* **2005**, The impact of metabolism on DNA methylation, *14*, R139-R147.
- (12) Stead, L. M.; Jacobs, R. L.; Brosnan, M. E.; Brosnan, J. T. *Advances in Enzyme Regulation* **2004**, Methylation demand and homocysteine metabolism, *44*, 321-333.
- (13) Purcell, E. M.; Pound, R. V.; Bloembergen, N. *Physical Review* **1946**, Nuclear magnetic resonance absorption in hydrogen gas, *70*, 980-987.
- (14) Emsley, J. W.; Feeney, J. *Progress in Nuclear Magnetic Resonance Spectroscopy* **1995**, Milestones in the first fifty years of NMR, *28*, 1-9.
- (15) Cohen, J. S.; Jaroszewski, J. W.; Kaplan, O.; Ruiz-Cabello, J.; Collier, S. W. *Progress in Nuclear Magnetic Resonance Spectroscopy* **1995**, A history of biological applications of NMR spectroscopy, *28*, 53-85.
- (16) Claridge, T. D. W. *High-Resolution NMR Techniques in Organic Chemistry*, 1 ed.; Pergamon: Oxford, 1999.
- (17) Sorland, G. H.; Seland, J. G.; Krane, J.; Anthonsen, H. W. *Journal of Magnetic Resonance* **2000**, Improved Convection Compensating Pulsed Field Gradient Spin-Echo and Stimulated-Echo Methods, *142*, 323-325.
- (18) Fulton, D. B.; Sayer, B. G.; Bain, A. D.; Malle, H. V. *Analytical Chemistry* **1992**, Detection and determination of dilute, low molecular weight organic compounds in water by 500 MHz proton nuclear magnetic resonance spectroscopy., *64*, 349-353.
- (19) Price, W. S.; Elwinger, F.; Vigouroux, C.; Stilbs, P. *Magnetic Resonance in Chemistry* **2002**, PGSE-WATERGATE, a new tool for NMR diffusion-based studies of ligand-macromolecule binding, *40*, 391-395.
- (20) Price, W. S. *Annual Reports on NMR Spectroscopy* **1999**, Water signal suppression in NMR spectroscopy, *38*, 289-354.
- (21) Finkelstein, J.; Martin, J. *J. Biol. Chem.* **1984**, Methionine metabolism in mammals. Distribution of homocysteine between competing pathways, *259*, 9508-9513.
- (22) Craig, S. A. S. *American Journal of Clinical Nutrition* **2004**, Betaine in human nutrition, *80*, 539-549.
- (23) Slow, S.; Lever, M.; Lee, M. B.; George, P. M.; Chambers, S. T. *International Journal of Biochemistry and Cell Biology* **2004**, Betaine analogues alter homocysteine metabolism in rats, *36*, 870-880.
- (24) Melse-Boonstra, A.; Holm, P. I.; Ueland, P. M.; Olthof, M.; Clarke, R.; Verhoef, P. *Am J Clin Nutr* **2005**, Betaine concentration as a determinant of fasting total homocysteine

- concentrations and the effect of folic acid supplementation on betaine concentrations, *81*, 1378-1382.
- (25) Lever, M.; George, P. M.; Dellow, W. J.; Scott, R. S.; Chambers, S. T. *Metabolism-Clinical and Experimental* **2005**, Homocysteine, glycine betaine, and N,N-dimethylglycine in patients attending a lipid clinic, *54*, 1-14.
  - (26) Mitchell, S. C.; Smith, R. L. *Drug Metabolism and Disposition* **2001**, Trimethylaminuria: the fish malodor syndrome, *29*, 517-521.
  - (27) Maschke, S.; Wahl, W.; Azaroual, N.; Boulet, O.; Crunelle, V.; Imbenotte, M.; Foulard, M.; Vermeersch, G.; Lhermitte, M. *Clinica Chimica Acta* **1997**, <sup>1</sup>H NMR analysis of trimethylamine in urine for the diagnosis of fish-odour syndrome, *263*, 139-146.
  - (28) Lever, M.; Sizeland, P. C. M.; Bason, L.; Hayman, C. M.; Chambers, S. T. *Biochimica et Biophysica Acta* **1994**, Glycine betaine and proline betaine in human blood and urine., *1200*, 259-264.
  - (29) Lever, M.; Sizeland, P. C. M.; Frampton, C. M.; Chambers, S. T. *Clinical Biochemistry* **2004**, Short and long-term variation of plasma glycine betaine concentrations in humans, *37*, 184-190.
  - (30) Dellow, W. J.; Lever, M.; Chambers, S. T.; Lunt, H.; Robson, R. A. *Diabetes Research and Clinical Practice* **1999**, Elevated glycine betaine excretion in diabetes mellitus patients is associated with proximal tubular dysfunction and hyperglycaemia., *43*, 91-99.
  - (31) Eisert, R.; Oftedal, O. T.; Lever, M.; Ramdohr, S.; Breier, B. H.; Barrell, G. K. *Marine Ecology Progress Series* **2005**, Detection of food intake in a marine mammal using marine osmolytes and their analogues as dietary biomarkers, *In press*.
  - (32) Slow, S.; Miller, W. E.; McGregor, D. O.; Lee, M. B.; Lever, M.; George, P. M.; Chambers, S. T. *European Journal of Clinical Nutrition* **2004**, Trigonelline is not responsible for the acute increase in plasma homocysteine following ingestion of instant coffee, *58*, 1253-1256.
  - (33) Nicholson, J. K.; Oflynn, M. P.; Sadler, P. J.; Macleod, A. F.; Juul, S. M.; Sonksen, P. H. *Biochemical Journal* **1984**, Proton-Nuclear-Magnetic-Resonance Studies of Serum, Plasma and Urine from Fasting Normal and Diabetic Subjects, *217*, 365-375.
  - (34) Bales, J. R.; Higham, D. P.; Howe, I.; Nicholson, J. K.; Sadler, P. J. *Clinical Chemistry* **1984**, Use of High-Resolution Proton Nuclear Magnetic-Resonance Spectroscopy for Rapid Multi-Component Analysis of Urine, *30*, 426-432.

- (35) Van, Q. N.; Chmurny, G. N.; Veenstra, T. D. *Biochemical and Biophysical Research Communications* **2003**, The depletion of protein signals in metabonomics analysis with the WET-CPMG pulse sequence, *301*, 952-959.
- (36) Podadera, P.; Areas, J. A. G.; Lanfer-Marquez, U. M. *Clinica Chimica Acta* **2005**, Diagnosis of suspected trimethylaminuria by NMR spectroscopy, *351*, 149-154.
- (37) Cartigny, B.; Azaroual, N.; Mille-Hamard, L.; Imbenotte, M.; Kintz, P.; Vermeersch, G.; Lhermitte, M. *Journal of Analytical Toxicology* **2002**, H-1 NMR urine analysis as an effective tool to detect creatine supplementation, *26*, 355-359.
- (38) Moolenaar, S. H.; Poggi-Bach, J.; Engelke, U. F. H.; Corstiaensen, J. M. B.; Heerschap, A.; De Jong, J. G. N.; Binzak, B. A.; Vockley, J.; Wevers, R. A. *Clinical Chemistry* **1999**, Defect in Dimethylglycine Dehydrogenase, a New inborn error of metabolism: NMR spectroscopy study., *45*, 459-464.
- (39) Hauet, T.; Gibelin, H.; Godart, C.; Eugene, M.; Carretier, M. *Clinical Chemistry and Laboratory Medicine* **2000**, Kidney retrieval conditions influence damage to renal medulla: Evaluation by proton nuclear magnetic resonance (NMR) spectroscopy, *38*, 1085-1092.
- (40) Grasdalen, H.; Belton, P. S.; Pryor, J. S.; Rich, G. T. *Magnetic Resonance in Chemistry* **1987**, Quantitative Proton Magnetic-Resonance of Plasma from Uremic Patients During Dialysis, *25*, 811-816.
- (41) Cornforth, J. W.; Henry, A. J. *Journal of the Chemical Society. Perkin Transactions 1* **1952**, The isolation of L-Stachydrine from the fruit of Capparis tomentosa., *1*, 601-602.
- (42) Fulton, D. B.; Sayer, B. G.; Bain, A. D.; Malle, H. V. *Analytical Chemistry* **1992**, Detection and Determination of Dilute, Low-Molecular-Weight Organic-Compounds in Water by 500-Mhz Proton Nuclear-Magnetic-Resonance Spectroscopy, *64*, 349-353.
- (43) Leyden, D. E.; Morgan, W. R. *Journal of Chemical Education* **1969**, Kinetics of proton exchange of trimethylammonium ion by N.M.R. Laboratory experiment, *46*, 169-171.
- (44) Lundberg, P.; Dudman, N. P. B.; Kuchel, P. W.; Wilcken, D. E. L. *Clinical Chemistry* **1995**, <sup>1</sup>H NMR determination of urinary betaine in patients with premature vascular disease and mild homocysteinemia, *41*, 275-283.
- (45) Slow, S.; McGregor, D. O.; Lever, M.; Lee, M. B.; George, P. M.; Chambers, S. T. *Clinical Biochemistry* **2004**, Dimethylglycine supplementation does not affect plasma homocysteine concentrations in pre-dialysis chronic renal failure patients, *37*, 974-976.

- (46) de Zwart, F. J.; Slow, S.; Payne, R. J.; Lever, M.; George, P. M.; Gerrard, J. A.; Chambers, S. T. *Food Chemistry* **2003**, Glycine betaine and glycine betaine analogues in common foods, *83*, 197-204.
- (47) Slow, S.; Donaggio, M.; Cressey, P. J.; Lever, M.; George, P. M.; Chambers, S. T. *Journal of Food Composition and Analysis* **2005**, The betaine content of New Zealand foods and estimated intake in the New Zealand diet, *18*, 473-485.
- (48) Bales, J. R.; Higham, D. P.; Howe, I.; Nicholson, J. K.; Sadler, P. J. *Clinical Chemistry* **1984**, Use of high-resolution proton nuclear magnetic resonance spectroscopy for rapid multi-component analysis of urine, *30*, 426-432.
- (49) Bell, J. D.; Lee, J. A.; Lee, H. A.; Sadler, P. J.; Wilkie, D. R.; Woodham, R. H. *Biochimica et Biophysica Acta* **1991**, Nuclear magnetic resonance studies of blood plasma and urine from subjects with chronic renal failure: identification of trimethylamine-N-oxide., *1096*, 101-107.
- (50) Cartigny, B.; Azaroual, N.; Mille-Hamard, L.; Imbenotte, M.; Kintz, P.; Vermeersch, G.; Lhermitte, M. *Journal of Analytical Toxicology* **2002**, <sup>1</sup>H NMR urine analysis as an effective tool to detect creatine supplementation, *26*, 355-359.
- (51) Choy, W. Y.; Au-Yeung, S. C. F.; Fan, S. Y.; Chiu, W. L. A. K.; Cockram, C. S. *Fresenius' Journal of Analytical Chemistry* **1998**, Quantitative determination of glucose in blood plasma by homonuclear proton decoupled water attenuation transverse relaxation Carr-Purcell-Meiboom-Gill (WATR-HDCPMG) NMR spectroscopy., *361*, 500-503.
- (52) Fan, S.; Choy, W. Y.; Lam, S. L.; Au-Yeung, S. C. F.; Tsang, L.; Cockram, C. S. *Analytical Chemistry* **1992**, Quantitative determination of glucose in blood plasma and in fruit juices by combined WATR-CPMG <sup>1</sup>H NMR spectroscopy., *64*, 2570-2574.
- (53) Grasdalen, H.; Belton, P. S.; Pryor, J. S.; Rich, G. T. *Magnetic Resonance in Chemistry* **1987**, Quantitative proton magnetic resonance of plasma from uremic patients during dialysis, *25*, 811-816.
- (54) Hauet, T.; Gibelin, H.; Godart, C.; Eugene, M.; Carretier, M. *Clinical Chemistry and Laboratory Medicine* **2000**, Kidney retrieval conditions influence damage to renal medulla: evaluation by proton nuclear magnetic resonance (NMR) spectroscopy., *38*, 1085-1092.
- (55) Holmes, H. C.; Snodgrass, G. J. A. I.; Iles, R. A. *European Journal of Pediatrics* **2000**, Changes in the choline content of human breast milk in the first 3 weeks after birth, *159*, 198-204.



- (56) Holmes, E.; Antti, H. *Analyst* **2002**, Chemometric contributions to the evolution of metabonomics: mathematical solutions to characterizing and interpreting complex biological NMR spectra, *127*, 1549-1557.
- (57) Keun, H. C.; Ebbels, T. M. D.; Antti, H.; Bollard, M. E.; Beckonert, O.; Schlotterbeck, G.; Senn, H.; Niederhauser, U.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. *Chemical Research in Toxicology* **2002**, Analytical Reproducibility in <sup>1</sup>H NMR-Based Metabonomic Urine analysis, *15*, 1380-1386.
- (58) Lee, M. B.; Storer, M. K.; Blunt, J. W.; Lever, M. *Clinica Chimica Acta* **2006**, Validation of <sup>1</sup>H NMR spectroscopy as an analytical tool for methylamine metabolites in urine, *365*, 264-269.
- (59) Lundberg, P.; Dudman, N. P. B.; Kuchel, P. W.; Wilcken, D. E. L. *Clinical Chemistry* **1995**, <sup>1</sup>H NMR determination of urinary betaine in patients with premature vascular disease and mild homocysteinemia, *41*, 275-283.
- (60) Maschke, S.; Wahl, A.; Azaroual, N.; Boulet, O.; Crunelle, V.; Imbenotte, M.; Foulard, M.; Vermeersch, G.; Lhermitte, M. *Clinica Chimica Acta* **1997**, <sup>1</sup>H-NMR analysis of trimethylamine in urine for the diagnosis of fish-odor syndrome, *263*, 139-146.
- (61) Moolenaar, S. H.; Poggi-Bach, J.; Engelke, U. F. H.; Corstiaensen, J. M. B.; Heerschap, A.; De Jong, J. G. N.; Binzak, B. A.; Vockley, J.; Wevers, R. A. *Clinical Chemistry* **1999**, Defect in dimethylglycine dehydrogenase, a new inborn error of metabolism: NMR spectroscopy study, *45*, 459-464.
- (62) Moolenaar, S. H.; Engelke, U. F. H.; Wevers, R. A. *Annals of Clinical Biochemistry* **2003**, Proton nuclear magnetic resonance spectroscopy of body fluids in the field of inborn errors of metabolism, *40*, 16-24.
- (63) Motta, A.; Romano, I.; Gambacorta, A. *Journal of Microbiological Methods* **2004**, Rapid and sensitive NMR method for osmolyte determination, *58*, 289-294.
- (64) Podadera, P.; Areas, J. A. G.; Lanfer-Marquez, U. M. *Clinica Chimica Acta* **2004**, Diagnosis of suspected trimethylaminuria by NMR spectroscopy, *351*, 149-154.
- (65) Wevers, R. A.; Engelke, U.; Heerschap, A. *Clinical Chemistry* **1994**, High-resolution <sup>1</sup>H-NMR spectroscopy of blood plasma for metabolic studies, *40*, 1245-1250.
- (66) Zuppi, C.; Messina, I.; Forni, F.; Rossi, C.; Pennacchietti, L.; Ferrari, F.; Giardina, B. *Clinica Chimica Acta* **1997**, <sup>1</sup>H NMR spectra of normal urines: reference ranges of the major metabolites, *265*, 85-97.

## Chapter 4

# Kinetic Measurements of BHMT

## CHAPTER 4: Kinetic Measurements of BHMT

### 4.1 Introduction

There are several methods available for the measurement of BHMT activity, but all suffer from various problems. Early assay techniques used a manometric method to measure CO<sub>2</sub> production, as an index of BHMT activity (they were measuring “thetin-homocysteine transmethylase”).<sup>1</sup> This approach was adopted when it was realised that the BHMT reaction, using the thetin substrates, results in lowered pH (the hydrogen ion on homocysteine is replaced with a methyl group, but is not absorbed by the thio-ether product). In the presence of sodium carbonate, the liberated acid generates CO<sub>2</sub>, which is measured in the headspace of the reaction.

Measuring the formation of methionine became a favoured technique when glycine betaine was employed as the substrate, because this reaction does not liberate hydrogen ions.<sup>2</sup> The procedure involved colorimetric analysis of the solutions with nitroprusside, which generates a coloured complex with methionine. This approach, however, can be hampered by a non-specific chromogen formation.

Other techniques for the measurement of BHMT have included a HPLC method, based on the detection of the methionine product.<sup>3,4</sup> In this method, the methionine is derivatised by 2,4-dinitrofluorobenzene or o-phthalaldehyde, forming a coloured (or fluorescent) adduct which can be easily detected from absorbance readings at 360 nm. Similar approaches are taken in the radiochemical assay, which uses radio labelled <sup>14</sup>C methyl groups on the betaine, which are passed onto the methionine product, and separated using ion-exchange chromatography. The tubes containing methionine are counted, and a measure of enzyme activity is derived from the radioactivity produced. Microbiological techniques for the detection of methionine production have

also been employed, using the growth of methionine auxotrophic bacteria on minimal media, supplemented with methionine produced by BHMT.<sup>5,6</sup>

In all three methods based on methionine production the final result is the only reading which is obtained, that is, the reaction is stopped by the addition of trichloroacetic acid, to precipitate the BHMT protein, prior to the reaction which quantifies the methionine produced. There are several problems with this method for deriving kinetic results. First, uncertainties in rates are greater if only one point is obtained – using a product-time graph reduces uncertainty in rate measurements. Second, there is no good way to ensure that the reaction is complete – or indeed if it has even begun. All the methods mentioned for derivatizing and separating the methionine from the reaction mixture take several hours of work, and problems with set-up of the experiment will not be apparent until the analysis is complete. More importantly, the reaction mixture is destroyed in each measurement, as the addition of trichloroacetic acid denatures the BHMT so no further work can be done. Third, there must be a commercially available source, or easy synthetic route, to obtain the required radioactive substrate. Even if a viable synthetic route is available, radioactive precursors must be sourced, and this is not always a simple task. Fourth, there is the complication of dealing with radioactive chemicals. Extra precautions must be taken to ensure that there is no contamination with the radioactive chemicals, and with extra health and safety issues.

There is one other method for assaying BHMT activity, developed by the Garrow group at the University of Illinois. A strain of *E. coli* has been developed (J5-3) which lacks a functional methionine synthase pathway, and so is a methionine auxotroph.<sup>6</sup> The growth of J5-3 is linearly dependent on methionine supplementation in their medium. If they are supplied with a plasmid coding for a functional BHMT protein and grown on medium lacking methionine, then they are able to augment their methionine supply and grow at a rate dependent on the BHMT activity. Effectively, a simple measure of the optical density (or the absorbance at 450 nm) gives a measure of the activity of the BHMT being coded by the plasmid introduced into the *E. coli*. This functional

complementation assay is particularly useful if assaying for mutations or for activity from novel substrates, but is unable to give kinetic results because the bacterial growth is slower than the rate of the enzyme reaction.

### 4.2 Kinetics of BHMT with Glycine Betaine

#### 4.2.1 Introduction

*In vitro* kinetic analysis of enzymes provides a useful insight into their mechanisms and biological function. The most simple analysis of enzyme kinetics is a form of the 'steady state approximation' or, as it is applied to enzymes, the Michaelis Menten equation:  $\text{Rate} = \frac{V_{\max} [S]}{K_m + [S]}$ .

This equation essentially defines the rate of the reaction (measured as either the appearance of products, or the loss of substrates) with two constants ( $V_{\max}$  and  $K_m$ ) and the concentration of substrates available to the enzyme at the time of the measurement.  $V_{\max}$  is the maximal rate that the enzyme would be capable of if the substrate were present at effectively infinite concentration (not including substrate-inhibited enzymes) and  $K_m$  is a combination of two rate constants (one for the rate of substrate binding, and the other for the rate of reaction once the substrate is bound). Once these have been measured for any given substrate in the enzyme, the Michaelis Menten constants can give clues into the biological function of the enzyme, as well as inferences about the binding site(s) and active site(s) of the protein, particularly if the constants obtained from different substrates are compared.

The assays described in **Section 4.1** are not easily adapted to operate on alternative substrates (either because they interfere with nitroprusside, or are not available in radiolabelled form), so  $^1\text{H}$ -NMR spectroscopy was identified as a potential assay tool. NMR spectroscopy has the advantage of being able to detect and quantify micromolar amounts of betaines, and produce a

measurement in under 2 min. Moreover, modern NMR spectrometers are fitted with temperature controlled sample probes, allowing kinetic analysis at different temperatures.

## 4.2.2 Materials and Methods

### 4.2.2.1 Materials

Glycine betaine monohydrate, its demethylated product DMG, trigonelline hydrochloride and homocysteine thiolactone were purchased from Sigma (St Louis, MO, U.S.A). Superdex 200 was purchased from Amersham Biosciences (UK).

### 4.2.2.2 Enzyme Purification

These procedures are all based on work described in **Chapter 2**. Liver BHMT was purified from an inbred Lewis rat after euthanasia by CO<sub>2</sub> asphyxiation. 12 g of liver was excised and homogenized with 3 volumes (w/v) of 20 mM potassium phosphate buffer containing 5 mM methionine and glycine betaine at pH = 7.5 (Buffer 1), in a manual glass homogenizer. The sample was centrifuged at 7000 *g* for 5 min, the supernatant was separated into 1.5 mL Eppendorf tubes and heat treatment for 10 min at 70 °C, followed by cooling on ice for 5 min and then centrifuged at 7000 *g* for a further 5 min. The supernatant (5.5 mL) was applied to a 450 mL Superdex G200 column, pre-washed and equilibrated with Buffer 1. Proteins were eluted with Buffer 1 at 1.1 mL/min and fractions collected every 13 mL.

Homocysteine (100 μmole) was added to each fraction, followed by incubation for 12 h at 4 °C. Fractions displaying a DMG methyl-group singlet by <sup>1</sup>H-NMR spectroscopy were collected and concentrated under pressure (40 psi) in a stirred concentration cell (Millipore) through a Diaflo ultrafiltration membrane (Amicon Corporation, MA). The concentrated sample was mixed with 20 mM potassium phosphate buffer (100 mL) at pH = 7.5 (Buffer 2) and reconcentrated three times

until no DMG, methionine or glycine betaine was found by  $^1\text{H}$ -NMR spectroscopy. The total volume was decreased during the buffer replacement, from 39 mL (total volume from three fractions containing BHMT activity) to 12.5 mL. Enzyme purity was assessed by SDS-PAGE electrophoresis. A single band at 45 kDa was observed [Chapter 2]. Total protein concentration was determined using the Bradford method (Bio-Rad), standardized with bovine serum albumin.

### *4.2.2.3 Preparation of Substrates*

L-Homocysteine was prepared for the assay using the method of Duerre and Miller.<sup>7</sup> L-Homocysteine thiolactone (0.7 mg) was dissolved in 2 M NaOH (0.4 mL), incubated for 5 min, then neutralized with saturated  $\text{KH}_2\text{PO}_4$  (0.6 mL).

### *4.2.2.4 $^1\text{H}$ -NMR Spectroscopy*

All  $^1\text{H}$ -NMR spectra were recorded on a Varian INOVA 500 instrument, at 37 °C in 5 mm NMR tubes with a 3 mm  $\text{D}_2\text{O}$  lock insert. For the measurements, a 90° radiofrequency pulse, with duration of 8.1  $\mu\text{s}$  was used. The delay between pulses was 5 s, acquisition time was 1.982 s. Eight transients were recorded for each sample, with 30,272 data points for each transient. Sweep width was set to 8,000 Hz. Shimming was completed using the field gradient autoshim macro. Water suppression was achieved using the DPFGE technique. All spectra were zero filled to 128k points, and then Fourier transformed. Phasing and baseline corrections were completed manually. The software used for these procedures was VNMR version 6.1C (Varian).

#### 4.2.2.5 Standard Line

A standard line for DMG and methionine was prepared, using 50, 100, 250, 500, 750  $\mu\text{M}$  and 1 mM solutions in Buffer 2. These were spiked with 5 mM *tert*-butanol (final concentration), and  $^1\text{H}$ -NMR spectra were obtained using the methods described in **Section 4.2.2.4**. The DMG peak integral to *tert*-butanol peak integral was calculated and plotted against DMG concentration to form the standard line.

#### 4.2.2.6 Enzyme Assay and Kinetics

Kinetic and endpoint assays were evaluated. For the kinetic assay, a solution of BHMT extract (protein = 97  $\mu\text{g/mL}$ ) was prepared containing a BHMT-saturating concentration of homocysteine (1 mM), glycine betaine (concentration varied from 0.05 to 2 mM) and 5 mM *tert*-butanol in Buffer 1 and placed in an NMR tube. DPGSE water suppression was used to remove the solvent peak. A pre-acquisition delay was used to automate acquisition, with delays between spectra acquisition set to 544 s (giving 10 min between spectra). A total of 12 spectra were acquired for each assay run. The  $^1\text{H}$ -NMR assay incorporates a short delay between mixing and spectrum acquisition; it took 160 s for the sample to be mixed, placed within a 5 mm NMR tube and the spectral parameters to be entered for each sample run. This was not a problem for BHMT analysis, since its activity is slow, with only 13.5  $\mu\text{mol}$  produced in one min under the assay conditions used. This activity was not detectable. Precision measurements were conducted on six solutions containing 2 mM glycine betaine and 1 mM homocysteine.

### 4.2.3 Results

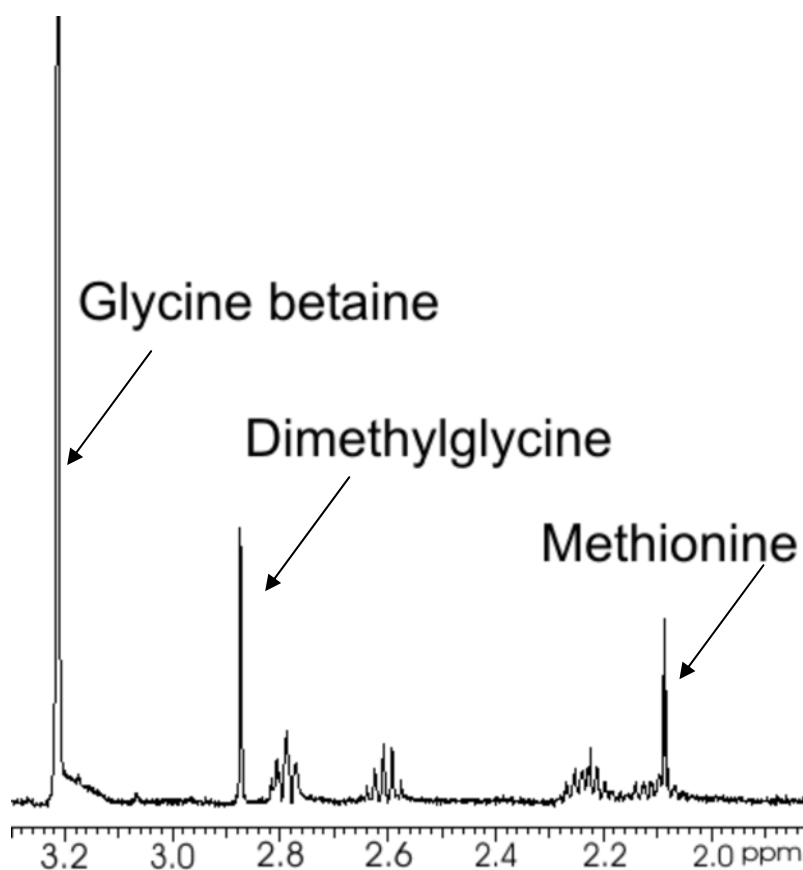
#### 4.2.3.1 NMR Spectra

After autophasing, the DMG peak was clearly visible at a chemical shift of 2.88 ppm, flanked by smaller, broad multiplets from homocysteine and methionine. The *tert* butanol signal



was clearly visible at 1.15 ppm, and the glycine betaine peak could be seen at 3.22 ppm [Figure 4.1]. Methionine appeared at 2.10 ppm, however this coincided with a broad resonance, and no kinetic analysis was performed using this peak. An example 10 h incubation sequence is shown in Figure 4.2.

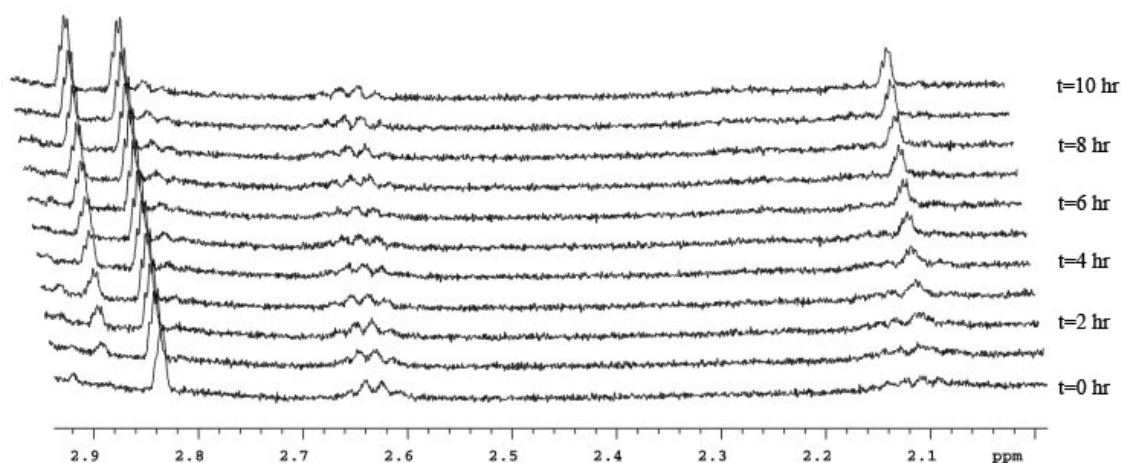
The large difference in concentration between glycine betaine, *tert*-butanol and DMG led to a deformity in the baseline of the spectra, which correlated to irregularities in the integrals obtained. This was corrected using the 'baseline correction' macros within the Varian software. This was also incorporated into the standard line for DMG, which was linear over six points (DMG concentration: 50  $\mu$ M – 2 mM,  $R^2=0.9991$ ).



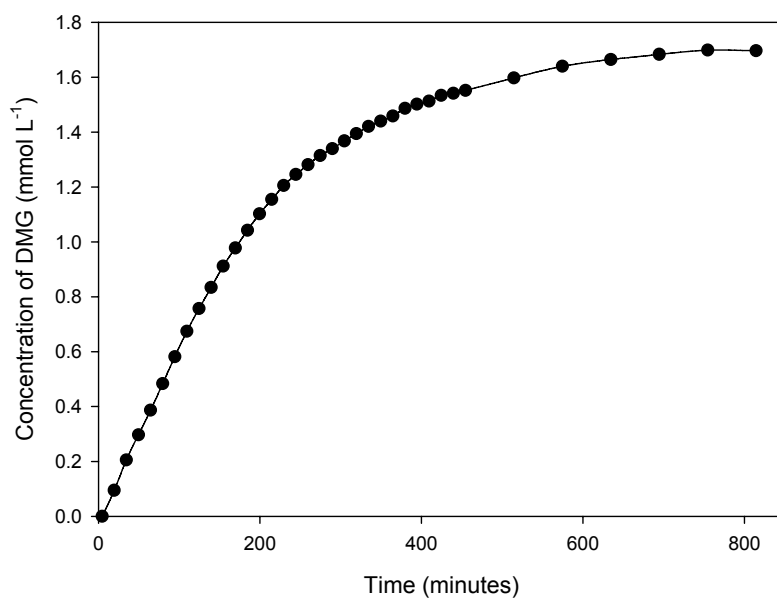
**Figure 4.1** A <sup>1</sup>H-NMR spectrum of the kinetic analysis performed on BHMT. Glycine betaine (3.21 ppm), DMG (2.86 ppm) and methionine (2.09 ppm) methyl singlets are all clearly visible in the spectrum, as well as multiplet signals from homocysteine (2.10, 2.60 ppm) and methionine (2.22, 2.79 ppm).

#### 4.2.3.2 Enzyme Reaction

Over a 14 h period, the concentration of DMG was observed to increase in a linear fashion for the first 150 min, reaching a plateau after 12 h [Figure 4.3]. For kinetic measurements, the data obtained over the first 2 h were used to derive the initial rate. The coefficient of variation for the measurement of DMG was 6.3% over six replicates (mean = 0.75 mM DMG) containing saturating levels of glycine betaine and homocysteine (10 mM and 2 mM, respectively – 300 nkatal). The limit of detection for the assay is 10  $\mu$ M of DMG, representing 6 nkatal under identical conditions. Activities below 6 nkatal gave signal to noise ratios lower than 3 for DMG in the  $^1\text{H}$ -NMR spectra.



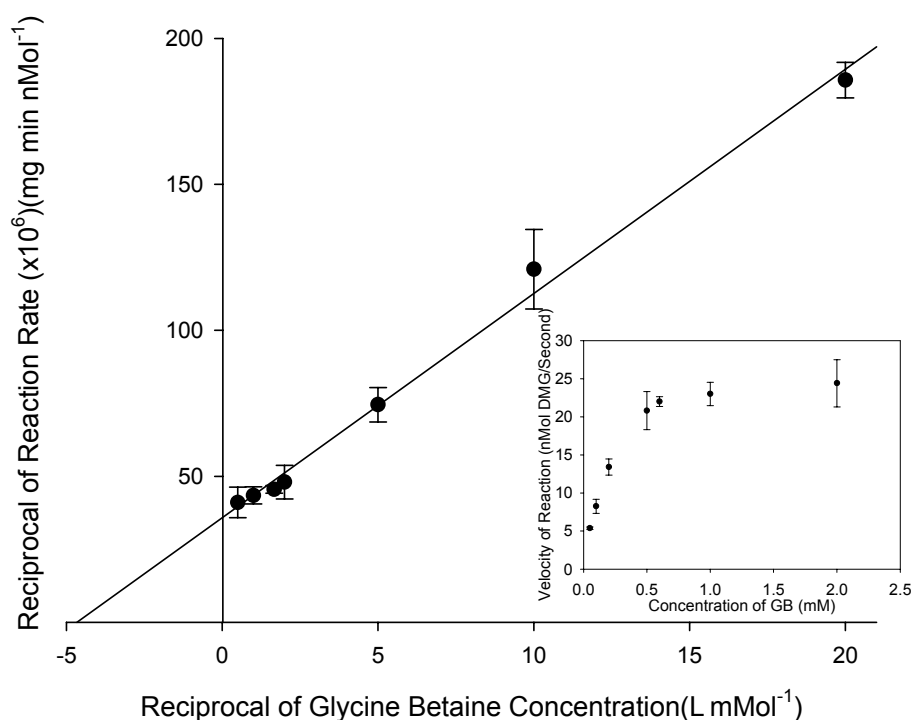
**Figure 4.2** Layered sequential  $^1\text{H}$ -NMR spectra (at 1 h intervals) showing homocysteine, methionine and DMG. Glycine betaine and homocysteine were initially at 2 mM, respectively (glycine betaine was off scale, and cut from this image). DMG (2.87 ppm above) and methionine (2.15 ppm above) are seen to increase over the incubation period.



**Figure 4.3** The BHMT reaction, as monitored by <sup>1</sup>H-NMR spectroscopy of the DMG peak.

#### 4.2.3.3 Michaelis Menten

The initial rate data using glycine betaine as a substrate were consistent with classical Michaelis Menten type kinetics, with a  $V_{\max} = 17 \pm 0.7 \text{ nmol min}^{-1} \text{ mg}^{-1}$  and a  $K_m = 0.19 \pm 0.03 \text{ mM}$  [Figure 4.4]. Estimates for  $V_{\max}$ ,  $K_m$  and uncertainties were determined using 'Enzfitter for Windows' (Biosoft V2.16.0.1).



**Figure 4.4** Lineweaver-Burk plot and Michaelis Menten plot (inset) of the BHMT reaction, as monitored by  $^1\text{H}$ -NMR spectroscopy. Error bars are derived from experimental variation.

### 4.3 The Endpoint Assay

#### 4.3.1 Introduction

One of the big problems when conducting enzyme assays, is determining the concentration of active protein in the sample. Normally the sample is purified as much as possible, as determined by specific activity, and the sample is then assumed to be 100% active protein. This can be confirmed by techniques like active site titration and SDS-PAGE but neither of these techniques will discern active protein from inactive protein.

BHMT is subject to feedback inhibition from DMG, assumed to be because of the formation of an abortive complex between BHMT, DMG and homocysteine [Chapter 1].<sup>8</sup> Because DMG is produced directly as a product of the betaine reaction, and is such a potent inhibitor of the enzyme, it offers an alternative method for the determination of active BHMT in the sample. This 'Endpoint

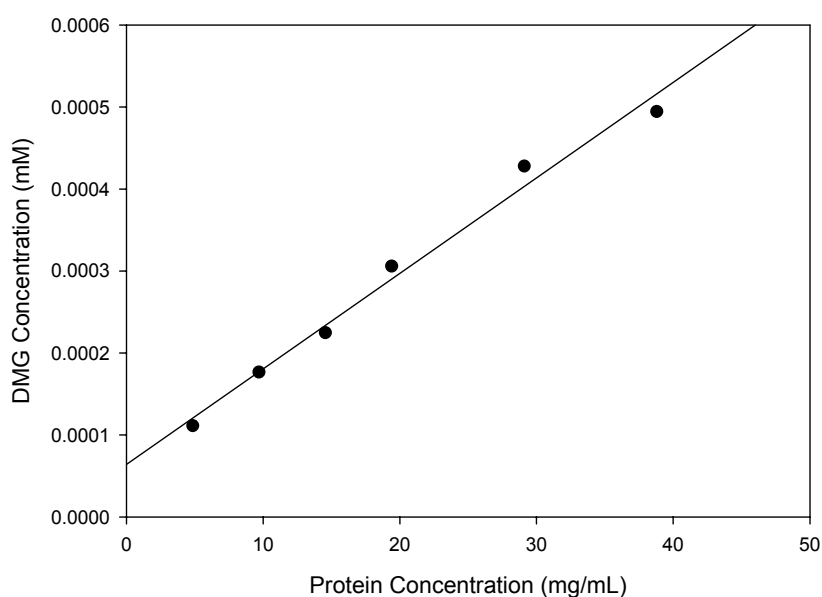
Assay' for BHMT can also be used to observe other potential substrates, and was employed in the work described in **Chapter 5**.

### 4.3.2 Method

Solutions containing 38.8, 29.1, 19.4, 14.6, 9.7 and 4.9 mg/mL BHMT were incubated with 2 mM glycine betaine and homocysteine for 24 h. The solutions were then analysed using <sup>1</sup>H-NMR spectroscopy (parameters as above in *Section 4.2.2*, without pre-acquisition delay) for DMG concentration. DMG was determined by comparing the DMG peak integral to a standard line, derived above in *Section 4.2.2.5*.

### 4.3.3 Results

The final DMG concentration was found to be dependent on the initial concentration of BHMT present in the solution. The linear relationship ( $R^2 = 0.986$ ) was used to measure the concentration of BHMT in an unknown solution [**Figure 4.5**]. The endpoint assay used any incubation time above 14 h [**Figure 4.3**] but had a greater sensitivity than the kinetic assay alone (the detection limit was 2 nkatal) and gave a CV = 5.3% (n = 6).



**Figure 4.5** Standard line showing the linear relationship between protein concentration (BHMT) and DMG concentration after 24 h of incubation ( $R^2 = 0.986$ ).

#### 4.4 Discussion

Since homocysteine has been identified as a cardiovascular risk factor,<sup>9-11</sup> its metabolism in mammals has been a subject of intense investigation. It is intimately linked with the metabolism of methyl groups, methionine and methyl donor availability.<sup>12</sup> In normal metabolism, homocysteine is either catabolized via cystathionine or it is methylated to form methionine. Most attention has focused on methylation by methionine synthase, requiring folate and B<sub>12</sub>-dependent enzyme systems, but increasing attention is being given to the role of BHMT in regulating the circulating homocysteine concentrations in mammals.<sup>13,14</sup>

The <sup>1</sup>H-NMR spectroscopic methods described here were able to deliver kinetic results, reproducibly (the CV on betaine and DMG is described in **Chapter 3**, but is 4.5% and 4.1% respectively) and, unlike other assays, offers several indices of activity (the disappearance of glycine betaine, or the appearance of methionine or DMG). NMR spectroscopy also offers the unique

ability of monitoring any potential alternate substrates, something which was explored for **Chapter 5**.

The kinetic constants obtained using these new techniques were comparable to those obtained using radiolabelled betaine, and the HPLC method. Various studies have reported  $K_m$  for glycine betaine to be 23,<sup>15</sup> 48<sup>16</sup> and 100  $\mu\text{M}$ <sup>17</sup>. The  $K_m$  for glycine betaine presented here (190  $\mu\text{M}$ ) is higher than these estimates, but the other methods for estimating BHMT activity have all suffered from an inability to trace the reaction as it progresses. **Figure 4.3** shows how slow the BHMT reaction is, when operating at  $V_{\text{max}}$ . Using saturating levels of glycine betaine (5 mM) the turnover rate for the enzyme was 0.8 /min.

The endpoint assay is a new application of the feedback inhibition by DMG to detect and measure the concentration of BHMT in an unknown sample. Similar techniques, applied to methionine, have been used by Ericson and others to identify the protein in crude homogenates.<sup>18</sup> These techniques will not give an accurate estimate of the BHMT concentration because there are other enzymes within the crude homogenate which are capable of using methionine as substrates.<sup>19</sup> It is likely, at least in the initial period, that those crude homogenates were using some of the produced methionine in secondary reactions. Furthermore, there are other methods for the production of methionine from homocysteine, via methionine synthase (E.C. 2.1.1.13, MS) [**Chapter 1**]. For these reasons, the production of methionine cannot be relied on as an accurate gauge of BHMT concentration or activity in crude homogenates.

The chance of interference in the DMG measurement is much lower than on methionine. There are only two known fates for DMG, demethylation by dimethylglycine dehydrogenase (DMGDH, E.C. 1.5.99.2) to form methylene-tetrahydrofolate [**Chapter 1**],<sup>20</sup> or excreted in the urine (impossible in crude samples). In mammals, the only known enzyme which uses DMG is DMGDH, localised on the inner membrane of the mitochondrion. In samples which have been homogenised

and solid material removed by centrifugation, it is unlikely that there are significant amounts of DMGDH present in the sample to interfere with the DMG production.

DMG was the best index of BHMT activity in crude and partially purified samples. In purified BHMT samples, there was no possibility of conflicting activity, so either methionine or DMG production, or betaine consumption could be used as a measure of relative activity. In crude extracts, it was unlikely that there would be any interfering enzyme activity which could confuse the interpretation of the results. Incubation times of 14 h were required, because of the slow turnover number of BHMT ( $0.8 \text{ min}^{-1}$ ).



## 4.5 Conclusion

The techniques described in this chapter were shown to be capable of reproducible kinetic results, with similar results to the already existing assays for BHMT. The NMR spectroscopic assay features all the benefits of the other assays, in a single analysis (kinetic results, on multiple substrates, with the ability to distinguish between alternative products, without the need for radiolabelled substrates) as well as, for the first time, being able to plot the activity of the enzyme over a time-course.

Furthermore, the endpoint assay described here could be useful in the analysis of multiple substrates and may find application clinically in the analysis of kidney or liver failure. When the kidney or liver is damaged, either through chemical or mechanical stress, often proteins which are normally found in the cytoplasm of the liver/kidney cells are leaked through the cell membrane into the plasma. The analysis of BHMT using this endpoint assay may establish another marker for this process, since BHMT is only found in the liver and kidney (in up to 1.6% of the soluble protein).<sup>15</sup> The endpoint assay would be a useful index for this process, given the ease of preparation and rapidity of NMR analysis. Unfortunately the >14 h incubation would make these measurements too cumbersome for routine clinical use.

## References

- (1) Maw, G. A. *Biochemical Journal* **1959**, Thetin-homocysteine transmethylase. The distribution of the enzyme, studied with the aid of trimethylsulfonium chloride as substrate, *72*, 602-608.
- (2) Borsook, H.; Dubnoff, J. W. *Journal of Biological Chemistry* **1947**, Methionine formation by transmethylation in vitro, *169*, 247-258.
- (3) Goeger, D. E.; Ganther, H. E. *Archives of Biochemistry and Biophysics* **1993**, Homocysteine-dependent demethylation of trimethylselenonium ion and selenobetaine with methionine formation., *302*, 222-227.
- (4) Lee, K. H.; Cava, M.; Amiri, P.; Ottoboni, T.; Lindquist, R. N. *Archives of Biochemistry and Biophysics* **1992**, Betaine:homocysteine methyltransferase from rat liver: purification and inhibition by a boronic acid substrate analog, *292*, 77-86.
- (5) Ericson, L. E. *Acta Chemica Scandinavica* **1960**, Betaine-homocysteine methyltransferases. III. The methyl-donor specificity of the transferase isolated from pig liver, *14*, 2127-2134.
- (6) Mulligan, J. D.; Laurie, T. J.; Garrow, T. A. *Journal of Nutritional Biochemistry* **1998**, An assay for betaine-homocysteine methyltransferase activity based on the microbiological detection of methionine, *9*, 351-354.
- (7) Duerre, J. A.; Miller, C. H. *Analytical Biochemistry* **1966**, Preparation of L-homocysteine from L-homocysteine thiolactone, *17*, 310-315.
- (8) Castro, C.; Gratson, A. A.; Evans, J. C.; Jiracek, J.; Collinsova, M.; Ludwig, M. L.; Garrow, T. A. *Biochemistry* **2004**, Dissecting the Catalytic Mechanism of Betaine-Homocysteine S-Methyltransferase by Use of Intrinsic Tryptophan Fluorescence and Site-Directed Mutagenesis, *43*, 5341-5351.
- (9) Boushey, C. J.; Beresford, S. A.; Omenn, G. S.; Motulsky, A. G. *Journal of the American Medical Association* **1995**, A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes, *274*, 1049-1057.
- (10) Nygård, O.; Nodrehaug, J. E.; Refsum, H.; Ueland, P. M.; Farstad, M.; Vollset, S. E. *New England Journal of Medicine* **1997**, Plasma homocysteine levels and mortality in patients with coronary artery disease, *337*, 230-236.
- (11) Welch, G. N.; Loscalzo, J. *New England Journal of Medicine* **1998**, Homocysteine and atherothrombosis, *338*, 1042-1050.

- (12) Park, E. I.; Garrow, T. A. *Journal of Biological Chemistry* **1999**, Interaction between dietary methionine and methyl donor intake on rat liver betaine-homocysteine methyltransferase gene expression and organization of the human gene., *274*, 7816-7824.
- (13) Lundberg, P.; Dudman, N. P. B.; Kuchel, P. W.; Wilcken, D. E. L. *Clinical Chemistry* **1995**, <sup>1</sup>H NMR determination of urinary betaine in patients with premature vascular disease and mild homocysteinemia, *41*, 275-283.
- (14) Slow, S.; Lever, M.; Lee, M. B.; George, P. M.; Chambers, S. T. *International Journal of Biochemistry & Cell Biology* **2004**, Betaine analogues alter homocysteine metabolism in rats, *36*, 870-880.
- (15) Garrow, T. A. *Journal of Biological Chemistry* **1996**, Purification, kinetic properties, and cDNA cloning of mammalian betaine-homocysteine methyltransferase, *271*, 22831-22838.
- (16) Finkelstein, J. D.; Harris, B. J.; Kyle, W. E. *Archives of Biochemistry and Biophysics* **1972**, Methionine metabolism in mammals: kinetic study of betaine-homocysteine methyltransferase., *153*, 320-324.
- (17) Skiba, W. E.; Taylor, M. P.; Wells, M. S.; Mangum, J. H.; Awad, W. M., Jr. *Journal of Biological Chemistry* **1982**, Human hepatic methionine biosynthesis. Purification and characterization of betaine:homocysteine S-methyltransferase, *257*, 14944-14948.
- (18) Ericson, L. E. *Acta Chemica Scandinavica* **1960**, Betaine-homocysteine methyltransferases. I. Distribution in nature, *14*, 2102-2112.
- (19) Tallan, H. H.; Cohen, P. A. *Biochemical Medicine* **1976**, Methionine adenosyltransferase: kinetic properties of human and rat liver enzymes, *16*, 234-250.
- (20) Meskys, R.; Harris, R. J.; Casaite, V.; Basran, J.; Scrutton, N. S. *European Journal of Biochemistry* **2001**, Organization of the genes involved in dimethylglycine and sarcosine degradation in *Artherobacter* spp. Implications for glycine betaine catabolism, *268*, 3390-3398.

## Chapter 5

# Alternative Methyl Donors with BHMT

## CHAPTER 5: Alternative Methyl Donors with BHMT

### 5.1 Methyl Donors in BHMT

The role of betaines in homocysteine metabolism is well described, but their mechanism of action is poorly understood. BHMT is the only known enzyme which is capable of demethylating glycine betaine. The fate of the DMG produced in this reaction is also well known in mammals; it is either excreted in the urine, or it is further demethylated to sarcosine, producing methylene-tetrahydrofolate (THF).<sup>1</sup> The sarcosine has a similar metabolic fate, being excreted, or further demethylated into glycine, forming another molecule of THF.<sup>2</sup> THF can also be used as a methyl donor, remethylating homocysteine into methionine via the enzyme methionine synthase.

It is known that the same transporter proteins that control glycine betaine concentration are also capable of transporting betaine analogues, such as arsenobetaine,<sup>3</sup> DMSP,<sup>4</sup> DMT,<sup>5</sup> trigonelline and proline betaine<sup>6-8</sup> (albeit in micro-organisms). The role of betaine analogues in BHMT activity and control has not been fully explored, except for a short paper by Ericson in 1960, in which he uses partially purified porcine liver BHMT, and exposes it to D,L-homocysteine and a variety of betaine analogues.<sup>9</sup>

Exposing enzymes to a number of substrate analogues and inhibitors is a powerful technique for getting information about the binding site of the enzyme. Ericson's experiment showed that a wide array of glycine betaine analogues were capable of transferring a methyl group to homocysteine, using microbiological detection of methionine to quantify the activity, after incubation for several hours.<sup>10</sup> The nitroprusside reaction was unsuitable for these

experiments, because it was found to give false positive measurements; some of the betaines would interfere with the formation of the coloured complex. The microbiological technique described by Ericson is not generally suitable, because it is not necessarily selective for methionine production and also because the growth of bacteria in the presence of trace, or low levels, of methionine is not linear. This means that any low levels of activity seen by Ericson are likely to actually reflect a much lower BHMT activity than reported. Furthermore, there is no guarantee that Ericson actually measures methionine, as there is no way of ensuring that some bacterial enzyme is not breaking down the substrates present in the reaction mixture and producing the results he records.

Ericson's results shows that porcine liver BHMT is sensitive to  $\alpha$ -substitution, increased chain length in the betaine (between the *tert*-amine moiety and the carboxylic acid) and substitution on the cationic centre. Substitution of the carboxylic acid moiety on glycine betaine for a carboxy-methyl ester, a carboxy-ethyl ester, or nitrile moiety retained some function (while lowering activity), but substitution of an amide moiety gave no activity at all. He did get traces of activity (13% vs glycine betaine) when the  $\alpha$ -methyl glycine betaine (alanine betaine) was incubated with BHMT.

Ericson's explanation for the decreased activity of alanine betaine was steric hindrance. He retested the same betaine in a 'swan mussel' (*Anodonta cygnea*, a freshwater bivalve mollusc which is relatively common in Britain) methyl transferase, and obtained a 45% activity following the same incubation period and conditions as before. He could not exclude the possibility that there were multiple methyltransferases in the mussel, which were contributing to a much higher activity with alanine betaine compared with porcine liver BHMT. However, the activity in the mussel was still significantly less than for glycine betaine alone. He did exclude the possibility of different BHMT/betaine ratios giving the anomalous results by

adding more alanine betaine to the porcine liver and getting no appreciable increase in activity.

Ericson also found that several betaines were inhibitors, including ergothionine (2-(2-mercapto-1*H*-imidazol-5-yl)-2-(trimethylammonio) acetate, structure shown below in **Section 5.1.3**) which was the first time this had been described. Ergothionine is a metabolite which is accumulated in the mammalian liver and red blood cells, but is of unknown function. He proposed that the three methyl groups located on the quaternary amine moiety in ergothionine might be good alkylating agents for homocysteine in BHMT, instead finding ergothionine to be a potent inhibitor, obtaining no activity when a ½ molar equivalent of glycine betaine was added to the reaction mixture.

Ericson's results for chain length indicated that, when an amine moiety was acting as the alkylating agent in BHMT, any increase in chain length (from glycine betaine to propiobetaine, butyrobetaine, and valerobetaine; structure shown below in **Section 5.1.3**) would yield no methionine. DMT was highly active, as was DMSP, and *S*-methyl methionine, each increasing in chain length by one carbon, respectively, DMT being the most active (1300 'units') versus 850 'units' for DMSP, 1200 'units' for *S*-methyl methionine and 100 'units' glycine betaine. In each case, the transfer of methyl groups from the thietins was inhibited by glycine betaine.

While these results are useful indicators of activity for further investigation, the use of microbiological assays should be treated with caution, because the rate of bacterial growth may not be linear at extremely low levels of activity, bacterial metabolism may lead to methionine production from a reaction which is not BHMT and the activity is not directly measured (except in the case of BHMT transformed methionine auxotrophs such as J5-3).<sup>11</sup> Nakajima, while working on fish analogues of BHMT from fish species, and using the nitroprusside reaction to quantify BHMT activity, found that DMSP gave more activity than

DMT and glycine betaine, in contrast to all other reported findings.<sup>12</sup> Furthermore, he reported activity from choline, dimethylsulfoxide and dimethylsulfone, which have been otherwise reported to be inactive in mammalian BHMT.<sup>12</sup> He also repeated these experiments on bovine, rat and chicken liver extracts, finding activity in all these samples, in comparable levels to the fish livers. All of Nakajima's experiments are conducted on crude extracts from livers, and there is likely to be metabolites present in the enzyme reactions which may give false positive readings. It is also possible that the BHMT activity Nakajima reported was actually a result of choline oxidation in the liver extracts he was using.

There have been other attempts to explore and describe the binding site of BHMT. Awad *et al* described a sulfur-alkylated homocysteine analogue (*S*-carboxybutyl L-homocysteine, CBH) which they suggest mimics the transition state of the BHMT reaction.<sup>13</sup> This model was the first to actually describe the reaction mechanism of BHMT, as one of direct methyl transfer between glycine betaine and zinc-bound homocysteine. The assays Awad and Skiba *et al* used for the CBH work were human liver BHMT extracts, and they used the radiochemical assay with <sup>13</sup>C-labelled glycine betaine; an assay technique which is much more specific for BHMT activity.<sup>13,14</sup> More recent work by Jiracek *et al* in synthesising BHMT inhibitors revealed structures similar to CBH as potent inhibitors, with IC<sub>50</sub> values in the low μM concentration range.<sup>15</sup> Their work has been conducted on recombinant human BHMT, which may have a different quaternary structure to purified liver BHMT [Chapter 2], but they also used the radiochemical assay. The only other inhibitor which has been described is a boronic acid derivative of glycine betaine, which was tested on a rat liver BHMT using an HPLC technique, and which uses *o*-phthalaldehyde to derivatise methionine to detect it.<sup>16</sup>

The only technique developed which is able to accurately measure the levels of activity unambiguously over all these betaine analogues, is the NMR spectroscopic assay described in Chapter 4. The microbiological assay will detect low levels of activity, but might not be linear



at low levels of BHMT activity, and so will not accurately quantify the initial velocity. Radiochemical assays require significant chemical research to develop synthetic strategies which will introduce radioactive methyl groups to the betaine analogues. Furthermore, the risks involved when handling radioactive substrates lead to them being avoided wherever possible. Inhibition studies would still be possible, using radioactive glycine betaine. The HPLC assay would require the development of fluorescent tags which would allow detection of the betaine analogues after the assay is completed, and the colorimetric assay may give false positive results, if the betaine analogue (particularly the sulfur analogues) reacts with nitroprusside. The NMR spectroscopic assay does not suffer from any of these problems. Furthermore, the NMR spectroscopic assay is the only assay technique that can continuously monitor the kinetics of the BHMT catalysed reaction.

The only research which has probed the binding of glycine betaine has been the IF work by Castro *et al.*<sup>17</sup> In this important paper, Castro and co-workers showed that there is no betaine binding to BHMT without the presence of homocysteine, that betaine and DMG did not bind to BHMT if methionine is present, and discovered two residues which appear to report the binding of glycine betaine (Trp-342 and 352 from the neighbouring BHMT subunit). These findings imply that there is a binding pocket for glycine betaine, but that this pocket is not available to glycine betaine until homocysteine has bound, perhaps through a conformational change. Trp-44 is known to be involved in betaine binding to BHMT from crystal structure observations of CBH-BHMT complexes.<sup>18</sup> It is also possible that the zinc-bound homocysteine forms a distinct part of the betaine binding site, and that the electron rich zinc-bound sulfur of homocysteine stabilises the partial positive charge on the methyl groups of glycine betaine.

### 5.1.1 Introduction

The potential for CVD drugs which use BHMT to lower circulating homocysteine (in the same way as folic acid, vitamin B<sub>12</sub> and glycine betaine are prescribed now) makes the investigation of methyl donors in BHMT an attractive prospect. Glycine betaine is limited in its capacity to lower homocysteine, partially because the *BHMT* gene is controlled by betaine and methionine concentrations,<sup>19-23</sup> because glycine betaine is subject to strong homeostasis,<sup>8,24-26</sup> self regulation by product inhibition via DMG,<sup>17,27</sup> and creates more methionine, which is converted via methylation reactions back into homocysteine.<sup>28,29</sup> These concepts are more fully explored in **Chapter 7, Section 7.1.2**. Alternative betaines may further reduce circulating homocysteine by not affecting the *BHMT* gene, and not producing DMG to inhibit BHMT activity. However, as Ericson discovered in 1960, mammalian liver BHMT is not active with all betaine analogues.<sup>9</sup> His experiments revealed that the active site of BHMT may be more discriminating than first thought (after it was found to work on DMT, DMSP and glycine betaine). A large library of betaine analogues was produced by Kelly Randal, a previous Ph.D. student in the Osmolyte Research group at Canterbury Health Laboratories, which provides a useful start to explore possible substrates and inhibitors of rat liver BHMT. Furthermore, the NMR spectroscopic assay is an ideal tool for the analysis of these betaine analogues, because the assay is simply adapted for use on these analogues and requires no special synthesis of <sup>13</sup>C labelled betaine analogues. Furthermore, it provides quantitative measurements quickly and simply which are directly related to BHMT activity, unlike the microbiological assay.

## 5.1.2 Method

### 5.1.2.1 Materials

Glycine betaine monohydrate, its demethylated product DMG, trigonelline hydrochloride and homocysteine thiolactone were purchased from Sigma (St Louis, U.S.A).

### 5.1.2.2 Synthesis of Betaines

#### Arsenobetaine ((carboxymethyl)trimethylarsonium bromide):

Trimethylarsine (0.5 mL, 1.124 g/mL,  $4.68 \times 10^{-3}$  moles) was dissolved under an inert argon atmosphere in toluene (5 mL). The solution was stirred and cooled on ice. Bromoacetic acid was recrystallised from a hot solution in hexanes and was dried *in vacuo*. One mole equivalent of the recrystallised bromoacetic acid (0.66 g,  $4.54 \times 10^{-3}$  moles) was dissolved in a further 5 mL of toluene and added dropwise to the stirred solution of trimethylarsine. A white precipitate was formed over the period of one minute, and the reaction was allowed to stir at room temperature for 16 h. The white solid was collected and dried *in vacuo*.

Yield: 0.965 g, 82%.

$^1\text{H-NMR}$  (500 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  1.77 (s, 9H,  $(\text{CH}_3)_3\text{-As}^+$ ), 3.21 (s, 2H,  $\text{CH}_2\text{-As}^+$ )

#### Phosphoniobetaine ((carboxymethyl)trimethylphosphonium bromide):

Bromoacetic acid (1.365 g,  $9.84 \times 10^{-3}$  moles) was dissolved in dichloromethane (10 mL), under an inert argon atmosphere and cooled with stirring over ice. Trimethylphosphine (1 g, 0.748 g/mL,  $9.83 \times 10^{-3}$  moles) was added drop-wise to the stirred solution and stirred at room temperature for 16 h. A white precipitate was formed and purified by washing with dichloromethane, and dried *in vacuo*.

Yield: 0.604 g, 28.6%.

$^1\text{H-NMR}$  (500 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  1.87 (s, 9H,  $(\text{CH}_3)_3\text{-P}^+$ ), 3.44 (s, 2H,  $\text{CH}_2\text{-P}^+$ )

Selenobetaine ((carboxymethyl)dimethylselenonium bromide):

Bromoacetic acid (1.806 g, 0.0130 moles) was dissolved in dichloromethane (10 mL) and cooled on ice under an inert argon atmosphere. Dimethylselenide (1 mL, 1.4077 g, 0.013 moles) was dissolved in a further 10 mL dichloromethane and added drop-wise to the bromoacetic acid solution, with constant stirring. The reaction mixture was allowed to warm to room temperature, and stirred for 15 h. The white precipitate was separated, washed with 10 mL dichloromethane and dried *in vacuo*.

Yield: 2.374 g, 73%

$^1\text{H-NMR}$  (500 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  2.66 (s, 6H,  $(\text{CH}_3)_3\text{-Se}^+$ ), 4.10 (s, 2H,  $\text{CH}_2\text{-Se}^+$ )

Dimethylselenopropanoate ((2-carboxyethyl)dimethylselenonium bromide)<sup>30</sup>:

Acrylic acid (5 mL, 5.255 g, 0.073 moles) was dissolved in ice cooled dichloromethane (50 mL). HCl gas was generated by the reaction of sulfuric acid and ammonium chloride, and bubbled into the solution. Dimethylselenide (5.6 mL, 7.951 g, 0.073 moles) was dissolved in ice cooled dichloromethane (20 mL) and introduced to the acrylic acid solution drop-wise, with the bubbling HCl gas. A white solid was formed almost instantly, and the solution was allowed to stir for 24 h at room temperature. The white solid was collected, washed with 10 mL dichloromethane and dried *in vacuo*.

Yield: 7.565 g, 47,6%

$^1\text{H-NMR}$  (500 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  2.68 (s, 6H,  $(\text{CH}_3)_3\text{-Se}^+$ ), 3.97 (t, 2H,  $\text{CH}_2\text{-Se}^+$ ), 3.45 (t, 2H,  $\text{CH}_2\text{-COO}^-$ )

#### 5.1.2.3 Preparation of Substrates

Homocysteine was prepared for the assay using the method of Duerre and Miller.<sup>31</sup> Homocysteine thiolactone (0.7 mg) was dissolved in 2 M NaOH (0.4 mL), incubated for 5 min, then neutralized with saturated  $\text{KH}_2\text{PO}_4$  (0.6 mL).

Betaine solutions were prepared to 10 mM in potassium phosphate buffer (20 mM, pH = 7.5). When mixed with the final incubation mixture, the betaine concentration was 2 mM.

#### 5.1.2.4 $^1\text{H}$ -NMR Spectroscopic Assay

All  $^1\text{H}$ -NMR spectra were recorded on a Varian INOVA 500 spectrometer, at 23 °C in 5 mm NMR tubes with a 3 mm  $\text{D}_2\text{O}$  lock insert. Spectra were recorded as previously described, using presaturation to suppress the water resonance.

#### 5.1.2.5 Enzyme Assay

For each alternative substrate, three separate tubes were prepared. Each tube contained 100  $\mu\text{L}$  of homocysteine solution, 100  $\mu\text{L}$  of potassium phosphate buffer (20 mM, pH = 7.5), 100  $\mu\text{L}$  of potassium phosphate buffer (20 mM, pH = 7.5) containing *tert*-butanol at 25 mM. One tube contained 100  $\mu\text{L}$  of 10 mM alternative betaine solution and 100  $\mu\text{L}$  of potassium phosphate buffer (20 mM, pH = 7.5), the second tube contained 100  $\mu\text{L}$  of 10 mM alternative betaine and 100  $\mu\text{L}$  of 10 mM glycine betaine, the third tube contained 100  $\mu\text{L}$  of 10 mM glycine betaine and 100  $\mu\text{L}$  of potassium phosphate buffer (20 mM, pH = 7.5). Activity was expressed as a ratio of integrals to *tert*-butanol (integral = 100), which had been shown to not inhibit BHMT.

### 5.1.3 Results

The results are reported as an average of the three replicates. For the activity, the initial integral of the betaine analogue is reported and the integral of the predicted demethylated product and methionine at 24 h also reported.

For inhibition, the DMG and methionine integrals are given as an index of methyl transfer from glycine betaine to homocysteine in the presence of the betaine analogue, recorded at  $t = 1$  h (data not shown below) and  $t = 24$  h.

#### *5.1.3.1 Activity Studies*

The results from this section reflect the tubes which contained either glycine betaine alone, or betaine analogue alone, and were designed to show any production of methionine in the absence of glycine betaine as a methyl donor. There were two measurements used to determine activity, being the production of methionine, and the production of the demethylated betaine analogue. Betaines which, when incubated with homocysteine and BHMT, produced methionine and a dimethylglycine analogue were assumed to be substrates of BHMT. The tables below show the methionine and demethylated betaine analogue integrals (where present) after 24 h incubation at 37 °C.

## 5.1.3.1.1 Chain Length

**Table 5.1** The effect of chain length on activity in rat liver BHMT. Activity is measured by the production of methionine and any demethylated betaine analogue after 24 h of incubation at 37 °C.

Betaine Analogue Name	Betaine Structure	Betaine Analogue Integral (t = 0 h)	Demethylated Product Integral (t = 24 h)	Methionine Integral (t = 24 h)
Glycine Betaine		46.8	7.5	5.2
Propiobetaine		40.7	0	0
Butyrobetaine		49.4	0	0
Valerobetaine		50.2	0	0
Caprobetaine		54.1	0	0
Trimethylsulfonium		32.3	0	5.7
Dimethylthetin		29.4	19.3	11.4
Dimethylsulfonio-propionate		26.7	16.7	3.8
Dimethylseleno-betaine		15.7	11.0	19.4
Dimethylseleno-propionate		13.8	7.7	16.7

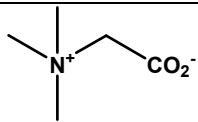
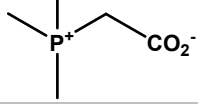
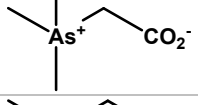
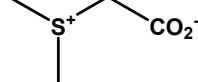
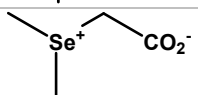
In accordance with Ericson's analysis,<sup>9</sup> when nitrogen was the heteroatom in the methyl donor, any increase in chain length resulted in no activity, while seleno- and sulfonio-betaines retained activity from aceto-thetins to propio-thetins (it is important to note that the thetin demethylated products measured above are likely to be complicated by the presence of

methionine peaks below them one of the methylene multiplets from methionine co-resonates with both methylthioacetate and methylthiopropiobetaine).

Trimethylsulfonium presented an interesting problem, in that there was no identifiable demethylated product of dimethylsulfide, or dimethylsulfoxide, only methionine and unreacted trimethylsulfonium. While it is unknown why no discernable demethylated product was observed, it is clear that trimethylsulfonium were capable of acting as a substrate of BHMT.

#### 5.1.3.1.2 Heteroatom Moiety

**Table 5.2** The effect of substitution of the nitrogen cationic centre in glycine betaine with another heteroatom on activity in rat liver BHMT. Activity was measured by the production of methionine and any demethylated betaine analogue after 24 h of incubation at 37 °C.

Betaine Analogue Name	Betaine Structure	Betaine Analogue Integral (t = 0 h)	Demethylated Product Integral (t = 24 h)	Methionine Integral (t = 24 h)
Glycine Betaine		46.9	7.4	5.5
Phosphoniobetaine		48.0	0	0
Arsenobetaine		52.8	0	0
Dimethylthetin		36.5	19.3	11.4
Selenobetaine		15.7	11.0	19.4

There was a clear difference in activity when the heteroatom moiety was changed. The group V heteroatom betaines did not transfer methyl groups if they were heavier than nitrogen, however the group VI heteroatoms did. It is worth noting that oxonium compounds



are used in organic synthesis and are powerful alkylating agents. There was no 'oxonium' betaine made, however, because it would not be stable in an aqueous environment.

#### *5.1.3.1.3 $\alpha$ -Substitution*

Out of the five  $\alpha$  substituted glycine betaine analogues (L-Alanine Betaine, D-Serine Betaine, D-Alanine Betaine, D,L-Valine Betaine, and D,L-Phenylalanine Betaine), none showed any methionine production, or formation of demethylated products. These  $^1\text{H}$ -NMR spectroscopic results confirmed Ericson's results, in that any  $\alpha$ -substitution in the betaine results in no activity. The work of Ericson was expanded to include stereospecific substitutions, but neither D or L alanine betaine, or D,L-valine betaine retained any activity with rat liver BHMT.

#### *5.1.3.1.4 N-Substitution*

Substitution on the quaternary amine moiety gave a loss of activity, possibly from steric interference, blocking the attack of homocysteine onto the methyl group. This is in contrast to Ericson's finding, in which there was some methyl transfer from *N*-ethyl, *N,N*-dimethyl betaine. The seven betaines tested were *N*-phenyl, *N,N*-dimethyl glycine; diethanol-thetin; triethanol betaine; tri-ethyl betaine; *N,N*-diethyl, *N*-methyl glycine; *N*-ethyl, *N,N*-dimethyl glycine; *N,N*-dimethyl, *N*-isopropyl glycine.

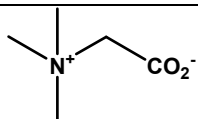
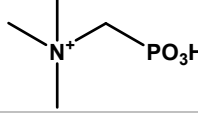
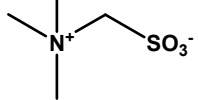
#### *5.1.3.1.5 Naturally Occurring Betaine Analogues*

In accordance with Ericson's results,<sup>9</sup> no activity was obtained from six of the other naturally occurring betaines which could potentially be found within the liver and could act as substrates (trigonelline, proline betaine, ectoine, betonicine, ergothionine and L-carnitine), although Ericson obtained traces of activity of methylation from ergothionine and L-carnitine

while the NMR spectroscopic assay detected none. There was activity from 10 mM *S*-methyl methionine, which produced 2.0 units of methionine in the absence of glycine betaine. Also, Garrow *et al* have detected traces of methylation from proline betaine in their *in vivo* bacterial assay.<sup>11</sup> There is no detectable methyl transfer from any of these betaines in the NMR spectroscopic assay.

#### 5.1.3.1.6 Substitution of Carboxylic Acid

**Table 5.3** The effect of substitution of the carboxylate moiety in glycine betaine for activity with rat liver BHMT. Activity was measured by the production of methionine and any demethylated betaine analogue after 24 h of incubation at 37 °C.

Betaine Analogue Name	Betaine Structure	Betaine Analogue Integral (t = 0 h)	Demethylated Product Integral (t = 24 h)	Methionine Integral (t = 24 h)
Glycine Betaine		46.8	7.5	5.2
Phosphonic Acid Betaine		298	1.2	2.0
Sulfonic Acid Betaine		448	0	2.2

As a consequence of the small quantities of sample available, only one experiment could be completed, and the necessary precursors for the synthesis of more analogues were not available. For these reasons, 10 mM phosphonic acid betaine and 15 mM sulfonic acid betaine were incubated with the BHMT solution, to ensure that any potential activity would be observed (all other conditions: homocysteine concentration, pH, etc, remained the same).

### 5.1.3.2 Inhibition/Allosteric Effects

These results compare the tubes which contained the betaine analogue of interest and glycine betaine after incubation. For these tubes, the levels of glycine betaine activity were compared. The results below reflect the glycine betaine integral at 24 h, as well as the methionine and DMG produced after 24 h. These were then compared to the integrals resulting from glycine betaine incubated in the absence of betaine analogue.

#### 5.1.3.2.1 Chain Length

**Table 5.4** The effect of chain length on glycine betaine activity in rat liver BHMT. Inhibition was measured by the production of DMG and methionine, compared to glycine betaine control after 24 h of incubation at 37 °C.

Betaine Analogue Name	Glycine Betaine Integral (t = 24 h)	DMG Integral (t = 24 h)	Methionine Integral (t = 24 h)	% Inhibition
Glycine Betaine	45.9	3.6	1.8	0
Propiobetaine	43.7	3.8	2.0	0
Butyrobetaine	44.2	3.7	1.9	0
Valerobetaine	44.3	3.3	2.1	0
Caprobetaine	44.8	3.1	2.0	0
Dimethylthetin	43.3	Uncertain; DMT and DMG co-resonant	8.3	Competitive Substrate
Dimethylsulfonio-propionate	45.5	Uncertain; DMSP and DMG co-resonant	4.0	Competitive Substrate
Dimethylseleno-betaine	47.8	0	24.9	100
Dimethylseleno-propionate	49.3	Uncertain; DMSeP and DMG co-resonant	16.6	Competitive Substrate

The analyses presented here support and expand the findings given in **Section 5.1.3.1** (the activity studies), that these betaines were not binding or fitting into the active site of BHMT. There was no apparent inhibition from any of the nitrogenous betaines with extended chain length. Furthermore, the betaines which showed rapid reaction led to greater production

of methionine. While these studies are not able to provide kinetic results, it is clear that the BHMT-active betaine analogues are competing for the same active site as glycine betaine. Furthermore, the seleno-betaine was so efficient at methylation that it did not allow for any methylation from glycine betaine, as it kinetically out-competed glycine betaine for BHMT.

#### 5.1.3.2.2 Heteroatom Moiety

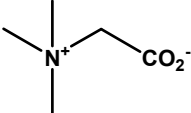
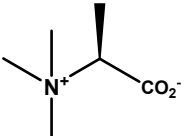
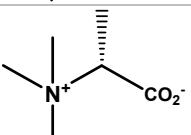
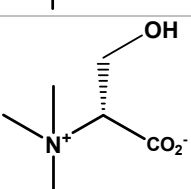
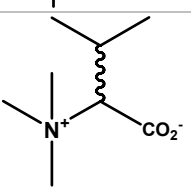
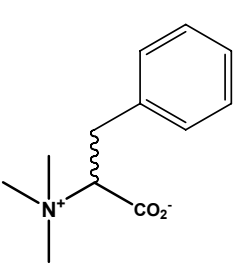
**Table 5.5** The effect of substitution of the nitrogenous cationic centre of glycine betaine with an alternative heteroatom on glycine betaine activity in rat liver BHMT. Inhibition was measured by the production of DMG and methionine (compared to glycine betaine control) after 24 h of incubation at 37 °C.

Betaine Analogue Name	Glycine Betaine Integral (t = 24 h)	DMG Integral (t = 24 h)	Methionine Integral (t = 24 h)	% Inhibition
Glycine betaine	46.8	7.5	5.2	
Phosphoniobetaine	48.4	7.4	5.0	0
Arsenobetaine	52.8	7.8	4.8	0
Dimethylthetin	43.3	Uncertain; DMT and DMG co-resonant	8.3	Competitive Substrate
Selenobetaine	47.8	0	24.9	100

The results for hetero-atom substitution corroborated the results from the activity studies in that the betaines that were not substrates were not acting as significant inhibitors. This implied that they were not binding in the active site, or that if they were binding, they were being easily displaced by glycine betaine.

5.1.3.2.3  $\alpha$ -Substitution

**Table 5.6** The effect of substitution on the  $\alpha$ -carbon of glycine betaine with larger side-chains on glycine betaine activity in rat liver BHMT. Inhibition was measured by the production of DMG and methionine, compared to glycine betaine control, after 24 h of incubation at 37 °C. (\* = Statistically significant difference compared to glycine betaine,  $p < 0.05$ ).

Betaine Analogue Name	Betaine Structure	Glycine Betaine Integral (t = 24)	DMG Integral (t = 24 h)	Methionine Integral (t = 24 h)	% Inhibition
Glycine Betaine		42.9	7.3	4.9	
L-Alanine Betaine		40.2	6.3	4.4	0
D-Alanine Betaine		38.8	6.9	5.1	0
D-Serine Betaine		40.2	7.2	5.2	0
D,L-Valine Betaine		42.2	6.9	4.9	0
D,L-Phenylalanine Betaine		42.3	8.8*	6.3*	0

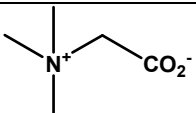
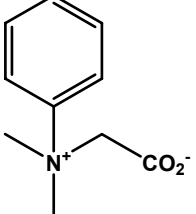
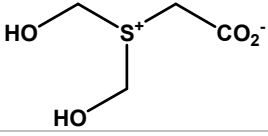
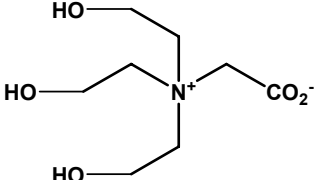
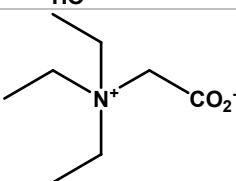
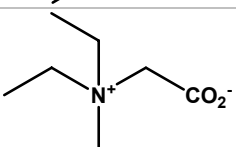
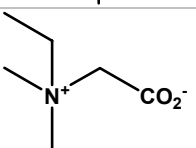
Evidence of  
Allosteric  
Affector

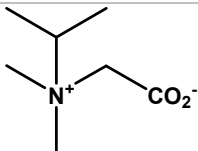
The results for  $\alpha$ -substituted betaines imply that alanine, serine and valine betaine were not acting as inhibitors, potentially because they were not binding to the enzyme. Phenylalanine betaine did provide an interesting result, in that it was not active as a

methylating agent (see section 5.1.3.1.3, above) but appeared to produce elevated methionine and DMG from glycine betaine.

#### 5.1.3.2.3 *N*-substitution

**Table 5.7** The effect of substitution of the methyl groups on the nitrogenous cationic centre of glycine betaine with larger side-chains on glycine betaine activity in rat liver BHMT. Inhibition was measured by the production of DMG and methionine, compared to glycine betaine control, after 24 h of incubation at 37 °C.

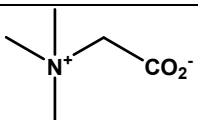
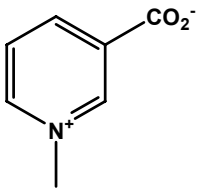
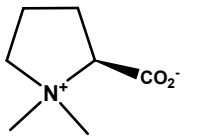
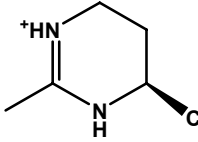
Betaine Analogue Name	Betaine Structure	Glycine Betaine Integral (t = 24 h)	DMG Integral (t = 24 h)	Methionine Integral (t = 24 h)	% Inhibition
Glycine Betaine		46.8	7.4	5.0	
<i>N,N</i> -Phenyl, <i>N,N</i> -dimethyl glycine		19.5	7.0	4.9	0
Diethanol-thetin		15.7 (CH <sub>2</sub> )	7.5	5.2	0
Tri-ethanol betaine		22.4 (N-CH <sub>2</sub> -)	7.0	5.2	0
Tri-ethyl betaine		37.5 (CH <sub>3</sub> )	7.3	4.9	0
<i>N,N</i> -diethyl, <i>N</i> -methyl glycine		13.3 (N-CH <sub>3</sub> )	7.7	5.0	0
<i>N</i> -ethyl, <i>N,N</i> -dimethyl glycine		24.7 (N-CH <sub>3</sub> )	7.4	4.9	0

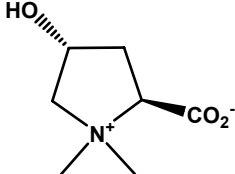
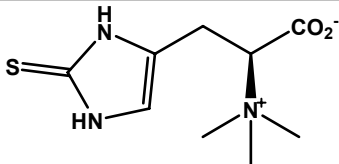
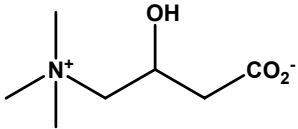
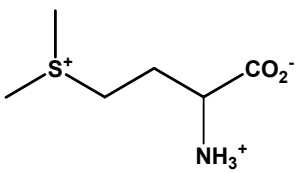
<i>N,N</i> -dimethyl, <i>N</i> -isopropyl glycine		14.7 (N-CH <sub>3</sub> )	7.6	4.8	0
---	---	------------------------------	-----	-----	---

Substitution of methyl groups on nitrogen with other, larger groups resulted in betaine analogues which did not appear to inhibit BHMT. This was in contrast to Ericson's findings,<sup>9</sup> which reveal some inhibition from triethyl-betaine and *N,N*-dimethyl, *N*-ethyl glycine betaine, although Ericson did not have the wide range of betaines available in the present work.

#### 5.1.3.2.4 Naturally occurring betaine analogues

**Table 5.8** The effect of naturally occurring betaine analogues on glycine betaine activity in rat liver BHMT. Inhibition was measured by the production of DMG and methionine (compared to glycine betaine control) after 24 h of incubation at 37 °C. (\* = statistically significant difference compared to glycine betaine,  $p < 0.05$ , <sup>S</sup>-methyl-methionine was at 10 mM, glycine betaine and homocysteine were both at 2 mM).

Betaine Analogue Name	Betaine Structure	Glycine Betaine Integral (t = 24)	DMG Integral (t = 24 h)	Methionine Integral (t = 24 h)	% Inhibition
Glycine Betaine		46.7	6.4	4.4	
Trigonelline		44.3	5.7	3.9	0
Proline Betaine		42.6	6.1	4.5	0
Ectoine		45.2	7.3*	6.0*	0 Evidence of Allosteric Affector

Betonicine		44.4	6.7	4.0	0
Ergothionine		45.6	5.7	4.2	0
L-Carnitine		44.0	5.9	3.8	0
S-methyl-methionine <sup>§</sup>		45.1	5.8	4.4	0

There appears to be no evidence that any of the naturally occurring betaines investigated above were acting as inhibitors of rat liver BHMT. The same effect that was observed with phenylalanine betaine was seen with ectoine. It did not act as a substrate, but appeared to activate methylation from glycine betaine, giving elevated DMG and methionine ( $p < 0.05$ ,  $n = 3$ ).

Oligomerisation is required for mammalian BHMT activity. Garrow *et al* have shown, using site-directed mutagenesis, that oligomerisation is required for stability of BHMT and also for catalytic activity.<sup>32</sup> Furthermore, a crystal structure resolved by Gonzalez *et al* established conformational changes upon substrate binding and release from the enzyme.<sup>33</sup> The binding of ectoine and phenylalanine betaine to the active site of one BHMT subunit may induce conformational changes in BHMT, resulting in more efficient methylation in the other active site(s). This hypothesis could be explored further with solution-state NMR studies, investigating diffusion of substrates into and out of the active site, and their effect on the large scale conformation on the BHMT structure.



## 5.2 Discussion

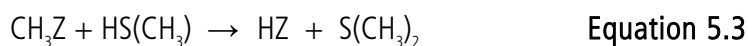
The initial integrals for glycine betaine and the betaine analogues varied between experiments for several reasons. In the case of the sulfonic acid and phosphonic acid analogues, there was so little available that accurate weight measurements were difficult. There is some inter-batch variation in the concentration of glycine betaine from the volume of samples investigated, which was minimised through replication of the experiments. Thetins, seleno-betaines and some of the other analogues also have reduced numbers of methyl groups, leading to reduced integral figures. Also, the solution behaviour of the betaines is likely to differ, and betaines are known to be excellent kosmotropes, which is likely to alter their hydration and also their NMR behaviour.<sup>34,35</sup>

The heteroatom substitution result was initially confusing, in that substitution of heavier hetero-atoms would result in an inactive betaine when in the group V elements, but would result in faster kinetics when in the group VI elements. To explain this result, molecular modelling of the nitrogen, arsenic, phosphorus, sulfur and selenium betaines was carried out by Dr Robert MacLagan in the Department of Chemistry, University of Canterbury.

To model the thermodynamics of the methyl transfer, *ab initio* electronic structure calculations of the N-CH<sub>3</sub> and N-H bonds in the gas phase were compiled. The same calculations were repeated with substitution of the nitrogen with phosphorus, arsenic, sulfur and selenium to compare the expected thermodynamics of the reactions.

Three separate methylation reactions were modelled at B3LYP/6-31G\*, MP2(FU) and G2/(MP2) levels of theory, incorporating vibrational and thermal corrections, scaled using scaling factors from the CCBDB database<sup>36</sup> and the GUASSIAN program. The reactions modelled were (where Z = heteroatom in the betaine being studied):





**Equations 5.1** and **5.2** are designed to model a simple radical type of transfer between the betaine in question and the homocysteine; essentially a concerted attack from homocysteine resulting in a bond breakage between the methyl group and the heteroatom. **Equation 5.3** is designed to better model the transfer to a thiol species. **Equation 5.4** is designed to model the methyl group leaving the betaine as a carbocation, resulting in a free methyl cation which the homocysteine sulfur can then attack. The reaction energies are calculated for Internal Energy ( $\Delta\text{U}$ , 0 K,  $\text{kJ mol}^{-1}$ ) and Enthalpy Change ( $\Delta\text{H}$ , 298 K,  $\text{kJ mol}^{-1}$ ). The results of these calculations are summarised in **Tables 5.9, 5.10, 5.11** and **5.12** below.

**Table 5.9:** Equation 5.1 calculation results,  $\Delta\text{U}$  and  $\Delta\text{H}$  for various betaines as they transfer a methyl radical group to a hydrogen atom.

Betaine ( $\text{CH}_3\text{Z}$ )	$\Delta\text{U}$ ( $\text{kJ mol}^{-1}$ )			$\Delta\text{H}$ ( $\text{kJ mol}^{-1}$ )
	B3LYP/6-31G*	MP2=FU/6-31G*	G2 (MP2)	G2 (MP2)
$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CO}_2^-$	-68.1	-8.6	-51.8	-49.5
$(\text{CH}_3)_3\text{P}^+\text{CH}_2\text{CO}_2^-$	-40.3	+25.2	-15.5	-15.3
$(\text{CH}_3)_3\text{As}^+\text{CH}_2\text{CO}_2^-$	-25.3	+38.3	-16.7	-16.8
$(\text{CH}_3)_2\text{S}^+\text{CH}_2\text{CO}_2^-$	-203.7	-158.5	-183.9	-182.7
$(\text{CH}_3)_2\text{S}^+\text{CH}_2\text{CH}_2\text{CO}_2^-$	-204.2	-151.9		
$(\text{CH}_3)_2\text{Se}^+\text{CH}_2\text{CO}_2^-$	-177.1	-133.2	-191.2	-190.3
$(\text{CH}_3)_4\text{N}^+$	-58.1	+0.4	-42.5	-40.0
$(\text{CH}_3)_3\text{S}^+$	-8.2	+45.7	+1.8	+2.9

**Table 5.10:** Equation 5.2 calculation results,  $\Delta\text{U}$  and  $\Delta\text{H}$  for various betaines as they transfer a methyl radical group to a methane group.

Betaine ( $\text{CH}_3\text{Z}$ )	$\Delta\text{U}$ ( $\text{kJ mol}^{-1}$ )			$\Delta\text{H}$ ( $\text{kJ mol}^{-1}$ )
	B3LYP/6-31G*	MP2=FU/6-31G*	G2 (MP2)	G2 (MP2)
$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CO}_2^-$	+0.6	+14.5	+21.9	+11.8
$(\text{CH}_3)_3\text{P}^+\text{CH}_2\text{CO}_2^-$	+28.4	+48.2	+49.2	+46.0
$(\text{CH}_3)_3\text{As}^+\text{CH}_2\text{CO}_2^-$	+43.4	+38.3	+48.0	+44.5
$(\text{CH}_3)_2\text{S}^+\text{CH}_2\text{CO}_2^-$	-135.0	-158.5	-119.2	-121.4
$(\text{CH}_3)_2\text{S}^+\text{CH}_2\text{CH}_2\text{CO}_2^-$	-135.5	-151.9		
$(\text{CH}_3)_2\text{Se}^+\text{CH}_2\text{CO}_2^-$	-108.4	-133.1	-126.5	-128.9
$(\text{CH}_3)_4\text{N}^+$	+10.6	+0.5	+22.2	+21.3
$(\text{CH}_3)_3\text{S}^+$	+60.5	+45.7	+66.5	+64.2

**Table 5.11:** Equation 5.2 calculation results,  $\Delta U$  and  $\Delta H$  for various betaines as they transfer a methyl radical group to a methane group.

Betaine ( $\text{CH}_3\text{Z}$ )	$\Delta U$ (kJ mol <sup>-1</sup> )			$\Delta H$ (kJ mol <sup>-1</sup> )
	B3LYP/6-31G*	MP2=FU/6-31G*	G2 (MP2)	G2 (MP2)
$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CO}_2^-$	-8.6	-2.8	+3.8	+4.4
$(\text{CH}_3)_3\text{P}^+\text{CH}_2\text{CO}_2^-$	+19.1	+30.9	+40.1	+38.6
$(\text{CH}_3)_3\text{As}^+\text{CH}_2\text{CO}_2^-$	+34.1	+44.0	+38.9	+37.1
$(\text{CH}_3)_2\text{S}^+\text{CH}_2\text{CO}_2^-$	-144.3	-152.7	-128.3	-128.6
$(\text{CH}_3)_2\text{Se}^+\text{CH}_2\text{CO}_2^-$	-144.8	-146.2		
$(\text{CH}_3)_4\text{N}^+$	-177.7	-127.4	-135.6	-136.3
$(\text{CH}_3)_3\text{S}^+$	+1.3	+6.3	+13.1	+13.9
$(\text{CH}_3)_3\text{S}^+$	+51.2	+51.5	+57.4	+56.8

**Table 5.12:** Equation 5.3 calculation results,  $\Delta U$  and  $\Delta H$  for various betaines as they transfer a methyl carbocation group.

Betaine ( $\text{CH}_3\text{Z}$ )	$\Delta U$ (kJ mol <sup>-1</sup> )			$\Delta H$ (kJ mol <sup>-1</sup> )
	B3LYP/6-31G*	MP2=FU/6-31G*	G2 (MP2)	G2 (MP2)
$(\text{CH}_3)_4\text{N}^+$	-427.4	-395.7	-362.9	-361.5
$(\text{CH}_3)_3\text{S}^+$	-377.5	-350.5	-318.6	-318.6

The results of these modelling studies reveal that there is a possible thermodynamic argument underpinning the results seen experimentally. The G2(MP2) is the most accurate calculation available, with an accuracy of  $\sim 10$  kJ/mol<sup>-1</sup>. **Tables 5.9, 5.10 and 5.11** all show an energy gap between the nitrogen betaines and the phosphorus/arsenic betaines (both in internal energy and enthalpy) which would explain why there is no activity observed for the heavier heteroatom betaines (phosponio-, and arsenio- betaines). While the models suggest that there is still an exothermic reaction taking place at G2(MP2) level, the models are only approximations, involving gas-phase reactions. It is likely that in the aqueous phase these approximations would be complicated by hydration (betaines are known to be powerful kosmotropic agents<sup>35</sup>) as well as the contribution of BHMT on their activity.

More striking is the absence of the same energy ‘jumps’ in the group VI compounds (sulfur and selenium). The calculations reveal that they are expected, under these approximations, to give highly exothermic reactions, over three times more exothermic than

the glycine betaine reaction. These results partially explain the increased kinetics seen in the enzyme reactions.

It is important to note initially that tertiary oxonium compounds are exceptional alkylating agents, and although there was no 'oxonium betaine' tested in the sequences above, oxonium ions are well described as powerful agents used in synthetic organic chemistry.<sup>37</sup> The oxonium betaine could not be tested because it would not be stable in the aqueous environment.

The effect of size on the active site in the enzyme can be explored by calculating the molecular dimensions of some of the active and inactive betaines and analogues tested in these experiments. For these calculations, basic MM2 (molecular mechanics 2) calculations were used to approximate the betaines size in X, Y and Z components. This approximate calculation was performed on glycine betaine, DMT, DMSP, propiobetaine, alanine betaine, phenylalanine betaine and ergothionine [Table 5.13].

These results show a clear size and shape difference between the nitrogenous and sulfonio betaines, as well as the inactive  $\alpha$ -substituted and naturally occurring ergothionine. Glycine betaine and DMT are approximately the same size (although glycine betaine is larger: a result of an extra methyl group), but DMSP and propiobetaine are quite different in dimensions (propiobetaine being larger in two dimensions) while phenylalanine betaine and ergothionine are substantially larger again.

**Table 5.13:** Results of MM2 calculations on selected betaines, revealing the approximate size of betaines compared.

Betaine	Longest Distance (Å)		
	X	Y	Z
Glycine betaine	5.5	3.9	3.9
DMT	5.7	3.9	3.2
Propiobetaine	7.0	4.1	3.9
DMSP	7.4	3.5	3.5
Alanine betaine	4.9	4.9	3.9
Phenylalanine betaine	5.4	7.7	5.7
Ergothionine	9.5	4.6	6.0

Combining the size and thermodynamic analyses reveals that both are likely to be influencing the substrate specificity of rat-liver BHMT. Larger betaines are not acting as substrates for BHMT because of some steric effect in the active site of the enzyme, revealed by the drop in activity from glycine betaine to propiobetaine, and mirrored in the DMT-DMSP activities. These facts also support the hypothesis that some of the larger betaines, particularly the naturally occurring betaines like ergothionine and L-carnitine, should not act as substrates in the enzyme.

When alternative heteroatoms are introduced into the betaine, the thermodynamics, and probably the reaction dynamics of the methyl transfer reaction are altered. The thermodynamic calculations above imply that there may well be an alternate mechanism for the transfer of methyl groups from glycine betaine and from DMT. Although these calculations hinge on a number of approximations, they strongly suggest the transfer of two electrons from the heteroatom-methyl bond to the heteroatom, resulting in a neutral heteroatom moiety. To infer kinetic results would be stretching the model beyond intended limits, since the modelling conducted did not predict reaction co-ordinates, but initial and final thermodynamic energies.

Arsenobetaine presents an interesting dilemma in that the models suggests it should not transfer a methyl group to homocysteine thermodynamically, yet *Rhizobium* and *Pseudomonas* sp. show clear evidence of methyl transfer.<sup>5,38,39</sup> There may be some coupling of another thermodynamically favourable reaction to drive this transfer in the species which can

demethylate arsenobetaine. It is also possible that there is some different mechanism for the demethylation of arsenobetaine in these species.

It is not immediately clear why propiobetaine is not a substrate for BHMT, while DMSP, and DMSeP are. While nitrogen based betaines are not good substrates for BHMT, thermodynamically, the sulfur and selenium betaines have much more favourable enthalpies, leading to much greater concentrations of methionine after incubation with BHMT and homocysteine. Increasing the chain length from a glycine betaine to a propiobetaine reduces activity in nitrogenous betaines to undetectable levels, while the same change in chain length decreases both the endpoint activities and the rates for DMSP and DMSeP. Because of their much greater alkylating strength, DMSP and DMSeP still act as substrates, giving higher concentrations of methionine. Propiobetaine does not act as a substrate, possibly because it does not form a favourable conformation within the enzyme, which adds to its low thermodynamic enthalpy and prevents it being a substrate.

From these results, a new model of betaine binding might be proposed. Castro *et al* proposed that there was no betaine binding site for BHMT before homocysteine is bound.<sup>17</sup> This concept developed from the observation of intrinsic tryptophan fluorescence (IF) in the presence, and absence of homocysteine, methionine, DMG and glycine betaine and combinations thereof, to investigate the mechanism of BHMT. The observation that there was no change in IF spectroscopy from the addition of DMG until homocysteine was previously bound lead to the proposal that the homocysteine was critical to the binding of betaine, possibly from conformational changes in the protein upon binding of homocysteine.

This hypothesis might be slightly modified to incorporate the data presented here. The methyl donor substrate might be binding only as a transition state. This would make homocysteine a crucial part of betaine binding (incorporating the data presented by Castro *et al*). Moreover, it allows for the observation that the carboxylate moiety is not required for

activity, and why there is activity also reported from thietins and other group VI heteroatom substitution. It might also explain why substitution on the heteroatom from three methyl groups would interfere with activity, since this substitution would also interfere with the formation of the transition state.

This reasoning might explain why trimethylsulfonium is a substrate, while tetramethylammonium is not; trimethylsulfonium is better stabilised by this homocysteine-zinc complex, forming an intermediate which will transfer a methyl group, while tetramethylammonium does not. The betaines all have carboxylate moieties, which will electrostatically bind to BHMT and then be stabilised by the homocysteine-zinc complex. It was demonstrated by Castro *et al* that DMG binds poorly to homocysteine saturated BHMT, probably because it is not stabilised by the homocysteine-zinc complex.<sup>17</sup> This complex formation would also be affected by steric crowding at the homocysteine-betaine interface, which may explain why some of the betaines presented in **Tables 5.4, 5.5, 5.10, and 5.11** are neither substrates nor inhibitors – they cannot form complexes with the homocysteine-zinc complex, and so have weak binding to BHMT.

This might be explored further by repeating the IF work of Castro *et al*, but including some of the alternative betaines presented in the results above.<sup>17</sup> BHMT is a slow enzyme, with a turnover rate of 0.8/min, so changes in IF as some of the betaine analogues which lack carboxylate functionality, but still are active in BHMT, are incubated with BHMT should give clues about the binding of betaines in BHMT.

This betaine-transition state binding model still allows for some betaines, such as ergothionine, to be inactive because of unfavourable steric interference, preventing it from reaching the active site of BHMT, although thermodynamic analysis would predict that there should be a reaction. It is still not clear why tetramethylammonium ions are not substrates, while trimethylsulfonium ions are able to transfer to homocysteine.

The enhanced betaine methylation from glycine betaine in the presence of ectoine and phenylalanine betaine was a surprise. No allosteric effector of BHMT has been described previously, and this finding suggests the presence of a possible cofactor for BHMT which has not been identified yet. This hypothesis might also explain the loss of activity from purified rat liver BHMT which was observed after anion-exchange chromatography, and also described by Lee *et al.*<sup>16</sup> If there is an unidentified cofactor for BHMT, this may be removed during purification, leading to an unstable protein. There is no added cofactor required for the recombinant BHMT activity, which would counter this hypothesis, unless it is a naturally occurring cofactor, produced in the *E. coli*, and tightly bound in the recombinant protein although, it would be expected that this cofactor would be present during x-ray crystallography studies. The absence of an unresolved region of electron density in the crystal structures implies there is no cofactor present.<sup>18,33,40,41</sup> There are several potential metabolites which are structurally related to ectoine and phenylalanine betaine: the nucleotides. It is possible that BHMT is activated by one or more of the nucleotides, perhaps adenosine, which is released by S-adenosyl-homocysteine as it is hydrolysed to free homocysteine. Perhaps this is evidence of an allosteric control system in BHMT which increases turnover *in vivo*. It is clear, however, that this requires further exploration.



### 5.3 Conclusion

The investigation of substrate specificity was greatly enhanced by the use of  $^1\text{H}$ -NMR spectroscopy. Unlike any previously existing methods, it provided multiple indices of activity, could independently measure most products and reactants in a single analysis and did not require specialist substrate preparation. Using the new assay, several naturally occurring betaines were rapidly assayed for activity in rat liver BHMT. Selenonio-betaine, dimethyl-selenoniopropionate, and trimethyltelluronium were all explored in the NMR spectrometer, and contrasted with Ericson's results from the 1960's, where he found traces of activity and inhibition from a number of betaines, including alanine betaine (activity) and carnitine, propiobetaine and N-substituted glycine betaine analogues (inhibition).

Sensitivity could be a complicating factor with the  $^1\text{H}$ -NMR spectroscopic assay as described, given the detection limits of 5  $\mu\text{M}$  methionine and DMG. Proline betaine has been described as a substrate by Mulligan *et al* but their detection technique was bacterial growth after supplementation with a methionine produced via BHMT. It is possible that their assay is more sensitive at lower concentrations than the  $^1\text{H}$ -NMR spectroscopic method, giving non-linear results at extremely low concentrations. Given the apparent sensitivity rat liver BHMT has to substitution in the methyl donor, it is likely that proline betaine is not a substrate for BHMT. It is possible that the *E. coli* used in the bacterial assay was capable of utilising the methyl groups on the proline betaine via some other pathway, giving a false-positive analysis. These complications were all resolved using the NMR spectroscopic assay.

The  $^1\text{H}$ -NMR assay for the first time allowed the direct observation of methyl transfer between betaines and homocysteine. The results presented by Ericson and Nakajima as they explored the role of betaine analogues in BHMT from mammals, fish and chickens all relied on indirect observation of activity (reactions allowing the observation of methionine were undertaken to measure BHMT activity, using either microbiological or nitroprusside

detection).<sup>9,12</sup> Any further exploration of the interactions between betaines and BHMT should include this <sup>1</sup>H-NMR assay.

## References

- (1) Binzak, B. A.; Vockley, J. G.; Jenkins, R. B.; Vockley, J. *Molecular Genetics and Metabolism* **2000**, Structure and analysis of the human dimethylglycine dehydrogenase gene., *69*, 181-187.
- (2) Brothers, V. M., 1975, p 134 pp.
- (3) Fujihara, J.; Kunito, T.; Kubota, R.; Tanabe, S. *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology* **2003**, Arsenic accumulation in livers of pinnipeds, seabirds and sea turtles: subcellular distribution and interaction between arsenobetaine and glycine betaine, *136C*, 287-296.
- (4) Kiene, R. P.; Hoffmann Williams, L. P.; Walker, J. E. *Aquatic Microbial Ecology* **1998**, Seawater microorganisms have a high affinity glycine betaine uptake system which also recognizes dimethylsulfoniopropionate, *15*, 39-51.
- (5) Pichereau, V.; Pocard, J.-A.; Hamelin, J.; Blanco, C.; Bernard, T. *Applied and Environmental Microbiology* **1998**, Differential effects of dimethylsulfoniopropionate, dimethylsulfonioacetate, and other S-methylated compounds on the growth of *Sinorhizobium meliloti* at low and high osmolarities, *64*, 1420-1429.
- (6) Lever, M.; Sizeland, P. C. B.; Bason, L. M.; Hayman, C. M.; Robson, R. A.; Chambers, S. T. *Clinica Chimica Acta* **1994**, Abnormal glycine betaine content of the blood and urine of diabetic and renal patients, *230*, 69-79.
- (7) Chambers, S. T.; Lever, M. *Nephron* **1996**, Betaines and urinary tract infections, *74*, 1-10.
- (8) Sizeland, P. C. B.; Chambers, S. T.; Lever, M.; Bason, L.; Robson, R. A. *Kidney International* **1993**, Organic osmolytes in human and other mammalian kidneys, *43*, 448-453.
- (9) Ericson, L. E. *Acta Chemica Scandinavica* **1960**, Betaine-homocysteine methyltransferases. III. The methyl-donor specificity of the transferase isolated from pig liver, *14*, 2127-2134.
- (10) Steele, B. F.; Sauberlich, H. E.; Reynolds, M. S.; Baumann, C. A. *Journal of Biological Chemistry* **1949**, Media for *Leuconostoc mesenteroides* P-60 and *Leuconostoc citrovorum* 8081, *177*, 533-544.
- (11) Mulligan, J. D.; Laurie, T. J.; Garrow, T. A. *Journal of Nutritional Biochemistry* **1998**, An assay for betaine-homocysteine methyltransferase activity based on the microbiological detection of methionine, *9*, 351-354.

- (12) Nakajima, K. *Nippon Suisan Gakkaishi* **1993**, Dimethyldithietin- and Betaine-homocysteine Methyltransferase Activities from Livers of Fish, Chicken, and Mammals, *59*, 1389-1393.
- (13) Awad, W. M., Jr.; Whitney, P. L.; Skiba, W. E.; Mangum, J. H.; Wells, M. S. *Journal of Biological Chemistry* **1983**, Evidence for direct methyl transfer in betaine:homocysteine S-methyltransferase, *258*, 12790-12792.
- (14) Skiba, W. E.; Taylor, M. P.; Wells, M. S.; Mangum, J. H.; Awad, W. M., Jr. *Journal of Biological Chemistry* **1982**, Human hepatic methionine biosynthesis. Purification and characterization of betaine:homocysteine S-methyltransferase, *257*, 14944-14948.
- (15) Jiracek, J.; Collinsova, M.; Rosenberg, I.; Budesinsky, M.; Protivinska, E.; Netusilova, H.; Garrow, T. A. *Journal of Medicinal Chemistry* **2006**, S-Alkylated homocysteine derivatives: new inhibitors of human betaine-homocysteine S-methyltransferase, *In Press*.
- (16) Lee, K. H.; Cava, M.; Amiri, P.; Ottoboni, T.; Lindquist, R. N. *Archives of Biochemistry and Biophysics* **1992**, Betaine:homocysteine methyltransferase from rat liver: purification and inhibition by a boronic acid substrate analog, *292*, 77-86.
- (17) Castro, C.; Gratson, A. A.; Evans, J. C.; Jiracek, J.; Collinsova, M.; Ludwig, M. L.; Garrow, T. A. *Biochemistry* **2004**, Dissecting the Catalytic Mechanism of Betaine-Homocysteine S-Methyltransferase by Use of Intrinsic Tryptophan Fluorescence and Site-Directed Mutagenesis, *43*, 5341-5351.
- (18) Evans, J. C.; Huddler, D. P.; Jiracek, J.; Castro, C.; Millian, N. S.; Garrow, T. A.; Ludwig, M. L. *Structure* **2002**, Betaine-Homocysteine Methyltransferase Zinc in a Distorted Barrel, *10*, 1159-1171.
- (19) Finkelstein, J. D. *European Journal of Pediatrics* **1998**, The metabolism of homocysteine: pathways and regulation., *157*, S40-S44.
- (20) Finkelstein, J. D.; Martin, J. J.; Harris, B. J.; Kyle, W. E. *Journal of Nutrition* **1983**, Regulation of hepatic betaine-homocysteine methyltransferase by dietary betaine, *113*, 519-521.
- (21) Finkelstein, J. D.; Harris, B. J.; Martin, J. J.; Kyle, W. E. *Biochemical and Biophysical Research Communications* **1982**, Regulation of hepatic betaine-homocysteine methyltransferase by dietary methionine, *108*, 344-348.

- (22) Finkelstein, J. D.; Kyle, W.; Harris, B. J. *Archives of Biochemistry and Biophysics* **1971**, Methionine metabolism in mammals. Regulation of homocysteine methyltransferases in rat tissue, *146*, 84-92.
- (23) Delgado-Reyes, C. V.; Garrow, T. A. *American Journal of Physiology* **2005**, High sodium chloride intake decreases betaine-homocysteine S-methyltransferase expression in guinea pig liver and kidney, *288*, R182-R187.
- (24) Grossman, E. B.; Hebert, S. C. *American Journal of Physiology* **1989**, Renal inner medullary choline dehydrogenase activity: characterization and modulation, *256*, F107-112.
- (25) Lever, M.; Sizeland, P. C. M.; Frampton, C. M.; Chambers, S. T. *Clinical Biochemistry* **2004**, Short and long-term variation of plasma glycine betaine concentrations in humans.
- (26) Lever, M.; Sizeland, P. C. B.; Bason, L. M.; Hayman, C. M.; Chambers, S. T. *Biochimica et Biophysica Acta* **1994**, Glycine betaine and proline betaine in human blood and urine, *1200*, 259-264.
- (27) McGregor, D. O.; Dellow, W. J.; Robson, R. A.; Lever, M.; George, P. M.; Chambers, S. T. *Kidney International* **2002**, Betaine supplementation decreases post-methionine hyperhomocysteinemia in chronic renal failure, *61*, 1040-1046.
- (28) Olthof, M. R.; Verhoef, P. *Current Drug Metabolism* **2005**, Effects of betaine intake on plasma homocysteine concentrations and consequences for health, *6*, 15-22.
- (29) Singh, R. H.; Kruger, W. D.; Wang, L.; Pasquali, M.; Elsas, L. J., II *Genetics in Medicine* **2004**, Cystathionine b-synthase deficiency: effects of betaine supplementation after methionine restriction in B6-nonresponsive homocystinuria, *6*, 90-95.
- (30) Samuelsson, L. M.; Randall, K.; Happer, D. A. R.; Peddie, B. A.; Lever, M. *Journal of Biochemistry Molecular Biology and Biophysics* **1998**, Effect of methyl substituents on the accumulation and osmoprotective effects of betaines, *2*, 43-50.
- (31) Duerre, J. A.; Miller, C. H. *Analytical Biochemistry* **1966**, Preparation of L-homocysteine from L-homocysteine thiolactone, *17*, 310-315.
- (32) Szegedi, S. S.; Garrow, T. A. *Archives of Biochemistry and Biophysics* **2004**, Oligomerization is required for betaine-homocysteine S-methyltransferase function, *426*, 32-42.
- (33) Gonzalez, B.; Pajares, M. A.; Martinez-Ripoll, M.; Blundell, T. L.; Sanz-Aparicio, J. *Journal of Molecular Biology* **2004**, Crystal Structure of Rat Liver Betaine

- Homocysteine S-Methyltransferase Reveals New Oligomerization Features and Conformational Changes Upon Substrate Binding, *338*, 771-782.
- (34) Moelbert, S.; Normand, B.; De Los Rios, P. *Biophysical Chemistry* **2004**, Kosmotropes and chaotropes: modelling preferential exclusion, binding and aggregate stability, *112*, 45-57.
- (35) Randall, K.; Lever, M.; Blunt, J. W.; Chambers, S. T. *Journal of Biochemistry, Molecular Biology and Biophysics* **1998**, Counteraction of urea denaturation by glycine betaine and other kosmotropes, *2*, 51-58.
- (36) CCBDDB; <http://srdata.nist.gov/cccbdb/vsf.asp>, Ed.
- (37) Louden, G. M. In *Organic Chemistry*, 3 ed.; Scanlan-Rohrer, A., Ed.; The Benjamin/Cummings Publishing Company, Inc: Redwood City, 1995, p 1390.
- (38) Pichereau, V.; Cosquer, A.; Gaumont, A.-C.; Bernard, T. *Bioorganic & Medicinal Chemistry Letters* **1997**, Synthesis of trimethylated phosphonium and arsonium analogs of the osmoprotectant glycine betaine; contrasted biological activities in two bacterial species, *7*, 2893-2896.
- (39) Jenkins, R. O.; Ritchie, A. W.; Edmonds, J. S.; Goessler, W.; Molenat, N.; Kuehnelt, D.; Harrington, C. F.; Sutton, P. G. *Archives of Microbiology* **2003**, Bacterial degradation of arsenobetaine via dimethylarsinoylacetate, *180*, 142-150.
- (40) Bose, N.; Greenspan, P.; Momany, C. *Protein Expression and Purification* **2002**, Expression of recombinant human betaine:homocysteine S-methyltransferase for x-ray crystallographic studies and further characterization of interaction with S-adenosylmethionine, *25*, 73-80.
- (41) Bose, N.; Momany, C. *Acta Crystallographica, Section D: Biological Crystallography* **2001**, Crystallization and preliminary x-ray crystallographic studies of recombinant human betaine-homocysteine S-methyltransferase., *D57*, 431-433.

# Comparative Studies of BHMT from Mammals, Aganthans and Other Species

## CHAPTER 6: Comparative Studies of BHMT from Mammals, Agnathans and Other Species

### 6.1 Introduction

Methionine (in the form of SAM) as a methyl donor is widely used in most organisms. In terms of evolutionary theory, it is logical to hypothesise that those enzyme systems that are associated with one-carbon metabolism, and the recycling of homocysteine back to methionine, would have evolved early in biological history. There is a common active site to a number of the methyltransferases associated with methionine synthesis, being two small motifs which contain a triad of cysteine residues. These cysteine residues all bind zinc in BHMT and cobalamin dependent methionine synthase enzymes.<sup>1,2</sup> Furthermore, the crystal structures of recombinant human BHMT and cobalamin-dependent MS from *Thermotoga maritima* have both been solved, and show remarkable similarities in *N*-terminal structure, a part of both proteins responsible for homocysteine binding.<sup>3,4</sup> BHMT and MS belong to the pfam 02574 family of thiol/selenol methyltransferases because of structural similarities.

BHMT is highly conserved in mammals; the human BHMT cDNA shares 88% identity with the porcine cDNA, and 94% amino acid identity with the porcine protein.<sup>5</sup> BHMT activity has been purified from horse, rat, pig and cow liver,<sup>6-8</sup> and has been detected in rhesus monkey lens tissue, human and chicken liver and in several other vertebrates.<sup>8-13</sup> BHMT activity has also been reported in several other kingdoms.<sup>14-17</sup> Notably, there is one report of BHMT activity from *Aspergillus nidulans*, a fungi, and appears to be the product of a single gene.<sup>14</sup>

Given this wide distribution of BHMT activity, and the highly conserved nature of the mammalian protein and gene, it can be hypothesised that other vertebrates will also have a



protein with BHMT activity. It could also be hypothesised that the BHMT protein from other vertebrates will be highly conserved. The most distant vertebrate from mammals is the Agnathan superclass of jawless fish, making them a logical point for investigation of these hypotheses.

The Agnathans, or 'jawless fish' are an ancient superclass which has two representative subclasses. The lamprey and hagfish are the two remaining representatives of the Cyclostomata class, and fall into the subclasses, Hypoartii and Hyperotreti respectively. Hagfish are salt water dwellers and have remained essentially unchanged morphologically for over 330 million years, and there are over 60 species. The lampreys are found within temperate coastal waters and brackish rivers, and are parasitic. Unlike hagfish, lampreys possess rudimentary vertebrae, which enable them to be classified as true vertebrates. There is still an active debate about the evolutionary relationship between hagfish, lampreys and other vertebrates.

BHMT is a zinc metalloenzyme<sup>10</sup> which comprises up to 2% of the soluble protein in the mammalian liver.<sup>3</sup> It has been found to be expressed in many mammalian kidney and livers including pig and human, and rat.<sup>10,18,19</sup> BHMT activity has also been described in Alfalfa sprouts (*Medicago marina*),<sup>20</sup> the fungus *Aspergillus nidulans*,<sup>14</sup> the cyanobacterium *Aphanothece halophytica*,<sup>21</sup> *Pseudomonads*,<sup>15,22</sup> and several extremophiles<sup>23</sup>, although there is recent discussion about the accuracy of the *Aspergillus* activity reports.<sup>24</sup> The BHMT protein appears to be highly conserved in mammals, having a 94% amino acid identity, with the human *BHMT* gene sharing 88% identity to the porcine gene.<sup>5</sup> BHMT has been purified to homogeneity from horse and rat liver.<sup>6</sup> Although BHMT activity is found in the liver and kidneys of most mammals, its distribution within other tissues seems to be species specific.<sup>8,25,26</sup> The BHMT protein has been identified as a lens crystallin in rhesus monkeys,<sup>12</sup> and in the pancreas of sheep.<sup>27</sup> In rats, there appears to be little or no activity in the kidney.<sup>25</sup> Mammalian BHMT also

shares similarities to *N*-terminal sequences of bacterial and eukaryotic methionine synthases.<sup>9</sup> Based on this sequence similarity, BHMT was allocated to the pfam 02574 family of thiol/selenol methyltransferases.<sup>9</sup>

This work was expanded slightly (and indirectly) by Nakajima in 1993, who explored the activities of thietin-homocysteine methyltransferase (THMT, shown in mammals to be BHMT) in rat, bovine and fish species (particularly carp, tuna and trout – but shark and other cartilaginous species were not included).<sup>28</sup> Nakajima found that each species tested possessed some form of THMT activity, which, when explored also displayed BHMT activity, and activity with *S*-methylmethionine ('vitamin U'), dimethylsulfone, dimethylsulfoxide and, in the case of yellowtail, rainbow trout and flounder, choline as well. Nakajima used the nitroprusside colorimetric assay for his BHMT activity measurements, which may be complicated by other metabolites remaining after the preparation of acetone powders. Ericson found no activity with choline in his BHMT assays,<sup>29</sup> however, Nakajima's experiments were performed using crude acetone-powder extracts of livers (and hepatopancreas where appropriate), so there could be some residual oxidation of the choline to betaine taking place.<sup>28</sup>

In a first step for a study of the evolution of BHMT, extraction, semi-purification and characterisation of substrate specificity of BHMT from lamprey and hagfish livers was undertaken, comparing this data to that obtained for rat BHMT [Chapter 5].<sup>30</sup>

## 6.2 Activity Studies

### 6.2.1 BHMT Activity in Lampreys

Ericson had reported BHMT activity in lamprey liver in 1960,<sup>8</sup> but did not further characterise the substrate specificity of the enzyme he reported. In order to confirm the presence of BHMT activity in agnathan liver, and to explore the activity in other classes of

marine organism (Cartilaginous fish), the NMR spectroscopic endpoint assay [Chapter 4] was used, with glycine betaine as a methyl donor to identify tissues for further analysis.

## 6.2.2 Method

### 6.2.2.1 Sources of BHMT

Active BHMT has been extracted from liver tissue directly,<sup>9,12,30-33</sup> as well as from liver acetone powders sourced commercially.<sup>25</sup> Hagfish are a common catch on commercial fishing boats, but are normally cast back because of their tendency to contaminate the deck of the boats with copious amounts of 'slime'.<sup>§</sup> Hagfish (*Eptatretus cirrhatus*) samples were obtained from a commercial fishing boat which had caught the fish in the Chatham Island area, immediately freezing the fish. The hagfish were partially defrosted, at room temperature under water, and the livers dissected as soon as defrosting allowed. The livers were then plunged into liquid nitrogen (-170 °C) until used in experiments.

Lamprey liver was sourced from a liver sample which had been stored at -80 °C for two years. No further lamprey liver samples could be obtained, so although BHMT activity was confirmed in the lamprey liver, analysis was restricted to hagfish liver.

Several commercial liver and kidney acetone powders were obtained and assayed for activity. In these experiments, rat liver BHMT was analysed from commercial liver acetone powder (Sigma, L-1380), along with bovine liver (Sigma, L-7751) and kidney (Sigma, K-4752), lemon-shark liver (Sigma, L-1130) and snail acetone powder (*Helix pomatia*, Sigma, S-9764).

### 6.2.2.2 Preparation of Samples

Acetone powders (100 mg) were suspended in 20 mM potassium phosphate buffer (1 mL, pH = 7.5). The samples were vortexed for 10 s, and then allowed to sit for 15 min at 4

---

<sup>§</sup> a defensive adaptation in hagfish, the mucous glands are a prominent feature along the lateral line of the hagfish body, and have earned them the common name of 'snot-fish'.

°C, before being centrifuged at 7000 g for 10 min. The supernatant was removed, and 100  $\mu$ L assayed for BHMT activity. A further 100  $\mu$ L was assayed for protein concentration using the Bradford method (Bio-Rad), standardized with bovine serum albumin [Chapter 2].

For the hagfish and lamprey liver samples, 100 mg of Liver was homogenised using a turreted homogeniser in 10 mL of 20 mM phosphate buffer (pH = 7.5) for 30 s. The samples were centrifuged at 7000 g for 10 min. The supernatant was removed and 100  $\mu$ L assayed for activity. A further 100  $\mu$ L was assayed for protein concentration as above.

#### *6.2.2.3 Preparation of Substrates*

L-Homocysteine was prepared for the assay using the method of Duerre and Miller.<sup>34</sup> L-Homocysteine thiolactone (0.7 mg) was dissolved in 2 M NaOH (0.4 mL), incubated for 5 min, then neutralized with saturated  $\text{KH}_2\text{PO}_4$  (0.6 mL).

#### *6.2.2.4 Activity Assay*

Hagfish liver extract (100  $\mu$ L), was incubated with 2 mM L-homocysteine, 2 mM glycine betaine and 5 mM tert-butanol. The final volume of the sample was 0.5 mL, which was placed in a sealed 5 mm NMR spectroscopy tube, and incubated in a 37 °C water bath for 24 h.

All  $^1\text{H}$ -NMR spectra were recorded on a Varian INOVA 500 instrument, at 23 °C in 5 mm NMR tubes with a 3 mm  $\text{D}_2\text{O}$  lock insert. For the measurements, a 90° radiofrequency pulse, with duration of 8.1  $\mu$ s was used. The delay between pulses was 5 s, acquisition time was 1.982 s. Eight transients were recorded for each sample, with 30,272 data points for each transient. Sweep width was set to 8,000 Hz. Shimming was completed using field gradient autoshim macros. Water suppression was achieved using a selective presaturating pulse on the water resonance for 3 s prior to the 90° pulse. All spectra were zero filled to 128k points, and

then Fourier transformed. Phasing and baseline corrections were completed manually. The software used for these procedures was VNMR version 6.1C (Varian).

The ratio of methionine produced to *tert*-butanol integrals was used as an index of activity. Specific activity was calculated by dividing the activity measured by the protein concentration.

### 6.2.3 Results

The mammalian liver samples all had BHMT activity [Table 6.1], while the bovine kidney, snail and shark liver acetone powders all failed to exhibit BHMT activity. The crude liver tissue extracts clearly have a higher specific activity than the acetone powders, which is likely to reflect some denaturing of BHMT in the acetone powders.

**Table 6.1** Results of BHMT activity studies. Hagfish and Lamprey results are from homogenised liver tissue.

Sample	Protein Concentration (mg/mL)	Met Integral (24 h)	Specific Activity
Rat Liver Acetone Powder	1.42	2.91	2.0
Rat Liver homogenate	22.8	75.1	3.3
Bovine Liver Acetone Powder	1.72	1.08	0.63
Bovine Kidney Acetone Powder	1.83	0	0
Lemon Shark Liver Acetone Powder	0.29	0	0
Snail 'Visceral Hump' Acetone Powder	3.00	0	0
Lamprey Liver	0.83	3.01	3.6
Hagfish Liver	2.12	7.19	3.4

## 6.3 Purification of Hagfish BHMT

### 6.3.1 Introduction

To characterise the hagfish BHMT, and compare it to rat BHMT, the hagfish extract was partially purified. The purification methods used were deliberately chosen to match the steps used for purification of the rat BHMT experiments and purification [Chapter 2], because these were known to be effective at purifying the rat protein, and to investigate similarities between the physical properties of the two proteins. Homogenisation and extraction into potassium phosphate buffer followed by heat shock treatment, size exclusion chromatography and concentration in a stirred concentration cell forms the basis of both schemes.

### 6.3.2 Method

Hagfish liver (12 g) was excised and homogenized with 3 volumes (w/v) of 20 mM potassium phosphate buffer at pH = 7.5 (Buffer 1), in a mechanical turret homogenizer (20 s, 5000 rpm). The sample was centrifuged at 7000 g for 5 min, the supernatant was separated into 1.5 mL Eppendorf tubes and heat shock treated for 2 min at 90 °C, followed by cooling on an ice slurry for 5 min and then centrifuging at 7000 g for a further 5 min. The supernatant (5.5 mL) was applied to a 450 mL Superdex G200 column, pre-washed and equilibrated with Buffer 1. Proteins were eluted with Buffer 1 at 1.1 mL/min and fractions collected every 13 mL.

The samples tubes had 100  $\mu$ M added to each one prior to fraction collection, and they were incubated for 12 hrs, before activity was determined using the  $^1\text{H}$ -NMR spectroscopic assay [Chapter 4]. All other procedures followed those set out in Chapter 2 for rat purification.

### 6.3.2 Results

Purification Table:

Purification type	Protein Concentration (mg/mL)	Volume obtained (mL)	Activity (Units/mL)	Specific Activity (Units/mg)	Percent Yield (%)	Purification
Crude homogenate	20.6	48	70.1	3.4	100	1
Heat Denaturation	2.07	28.5	43	20.8	61.3	6.1
Size Exclusion Chromatography	0.230	37.5	23	100	0.1	29.4

## 6.4 *N*-terminal Sequencing

### 6.4.1 Introduction

In an attempt to confirm that the hagfish BHMT activity seen was due to a protein that is homologous to BHMT, *N*-terminal sequencing was conducted on the 45 kDa band seen in the SDS-PAGE gel, suspected to be the BHMT subunit. There is no known hagfish BHMT cDNA, but it could be expected that, given the conserved nature of mammalian, chicken and zebra-fish BHMT sequence (from cDNA data), the hagfish BHMT sequence could be expected to have some conserved nature around the *N*-terminal protein sequence [Figure 6.2].

### 6.4.2 Method

Purified hagfish BHMT (concentrated to 1 mg/mL) was analysed using gradient SDS-PAGE electrophoresis (5-20 %, Sigma) and stained for 1 h with Coomassie Blue stain. The gel was destained using 10% acetic acid in methanol, washed twice for 2 h each time. A second SDS-PAGE was conducted using the same hagfish BHMT extract and Sigma Wide markers then electro-blotted at 100 mA constant current (voltage ~45 V) for 15 h to PVDF

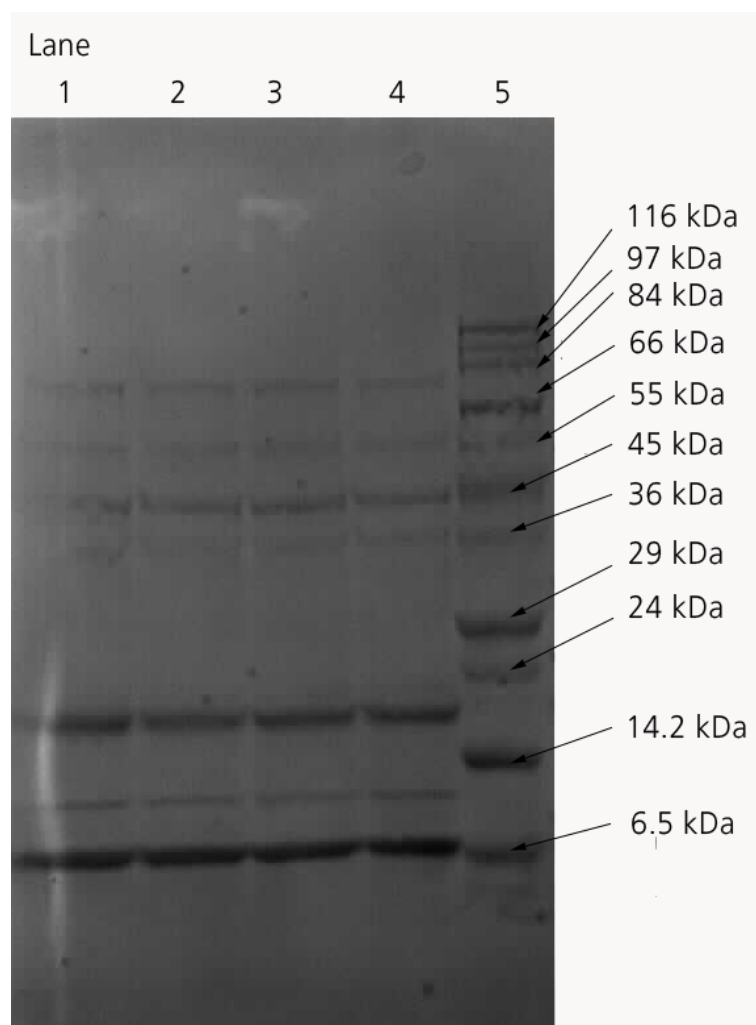
membrane, and stained for one minute with amido black stain, followed by washing twice with 10% acetic acid in water.

The 45 kDa band was identified by comparison with Sigma wide-range markers (M 4038, Sigma) and the ~45 kDa band on the PVDF blot was excised. The sample was submitted to the Protein Microchemistry Facility in the Department of Biochemistry at the University of Otago [Figure 6.1].

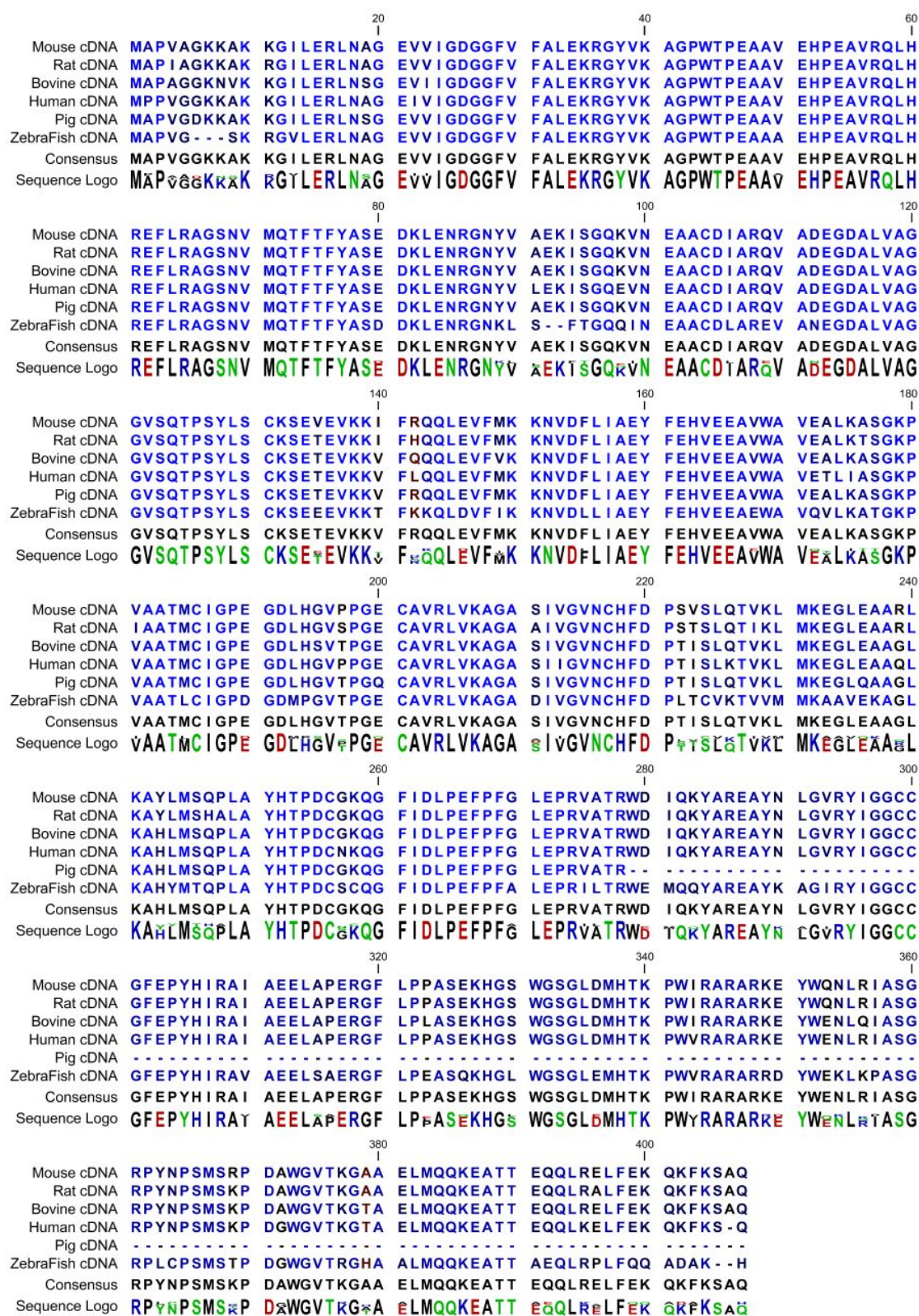
### 6.4.3 Results

Despite an intense 45 kDa band being excised in the hagfish extract, no sequence was obtained despite two attempts. This is possibly because the *N*-terminal amino acid is blocked by acetylation, which is preventing Edmann degradation. Attempts to deblock the hagfish sequence using an Acid-Alcohol-Heat process *in situ* (an in-house method used in the Protein Microchemistry Facility) did not result in any improved sequence data.





**Figure 6.1** Hagfish BHMT PVDF blot showing hagfish extract, post size-exclusion chromatography (lanes 1-4), Sigma Wide-Range markers (lane 5).



**Figure 6.2** Alignment of mouse, rat, bovine, human, porcine and zebra-fish BHMT amino acid sequences, as predicted from cDNA data (obtained via the NCBI databases). Consensus sequences and a graphical representation of the sequence is included below the alignment. Alignment was performed using the CLC Gene Workbench package (Version 1.0.2).

Initially, a band of ~20 kDa was excised and sequenced, giving “D S L S K E Q V E S A V D E A L D K L N K Q Q V S T R K L A L A E E”. Blast searching for this sequence gave a positive ( $p = 6.8 \times 10^{-10}$ ) ID for “Hematopoietic antimicrobial peptide-37 precursor”, or “Hematopoietic antimicrobial peptide-29 precursor”.<sup>35</sup>

## 6.5 Comparison of Rat and Hagfish BHMT Activity

### 6.5.1 Background to Comparison Studies

Ericson explored the distribution of BHMT across various classes of animal.<sup>8</sup> Using the production of methionine as a measure of BHMT activity, he showed that extracts from all of the vertebrates he tested remethylated homocysteine using glycine betaine. In two papers that followed, he then explored the properties of the mammalian (pig) enzyme, including the substrate specificity (presented in **Chapter 5**). He noted that the only non vertebrate animal which had BHMT activity was the fresh-water mussel *Anodonta cygnea*, insects and arachnids having no activity in his crude extracts.

In **Section 6.2** above, activity was found in mammalian (rat) liver acetone powders, but was absent in shark acetone powder, a species which was absent from Ericson’s research. Snail acetone powder also lacked BHMT activity. There has been little characterisation of BHMT activity in animals beyond the class of mammals. Ericson briefly attempted to show that, in some of the BHMT activities he observed, DMSP would replace glycine betaine as a methyl donor, and choline would not. Furthermore, he showed that DMG inhibited the reactions, but choline did not affect the methyl transfer.

To expand this data and make a brief comparison of BHMT from hagfish and rats, the substrate specificity of partially purified hagfish BHMT was explored. Substrates with activity in rat extracts were incubated with purified hagfish extract, and compared to glycine betaine.

Arsenobetaine was included in this experiment, because it is known to be a naturally occurring marine betaine analogue and is reportedly a substrate for *Paenibacillus* sp.<sup>22,36-40</sup>

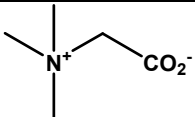
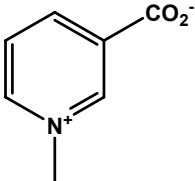
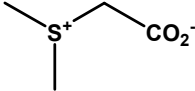
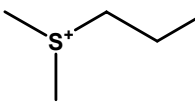
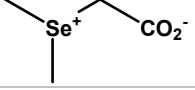
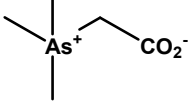
### 6.5.2 Method

Potential substrates were evaluated for activity and inhibition in two separate experiments. Purified hagfish BHMT (100  $\mu$ L) was incubated at 37 °C for 24 h with 2 mM homocysteine and either 10 mM trigonelline, L-proline betaine, DMT, DMSP, selenobetaine or arsenobetaine. These solutions were prepared containing an additional 10 mM glycine betaine, incubated identically to the tubes containing no glycine betaine and compared with control solutions, containing glycine betaine (10 mM) and homocysteine (2 mM) but no betaine analogues. The relative activity in each solution was evaluated by monitoring production of DMG (for solutions containing glycine betaine), and methionine at 1 and 24 h using the <sup>1</sup>H-NMR spectroscopic assay [Chapter 4].

## 6.5.3 Results

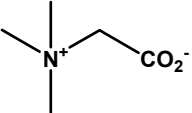
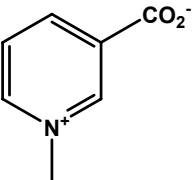
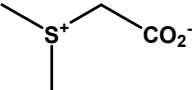
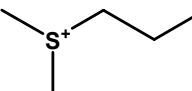
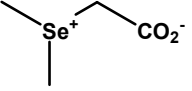
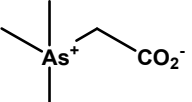
## 6.6.3.1 Activity Studies

**Table 6.3** Results of the activity studies in hagfish BHMT extract.

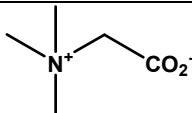
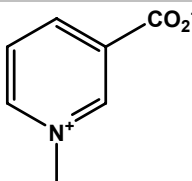
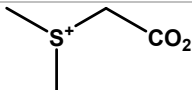
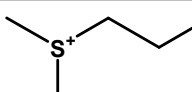
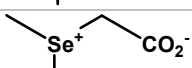
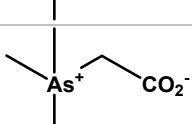
Betaine Name	Betaine Structure	Betaine Integral (t = 0)	Demethylated Product Integral (t = 24 h)	Methionine Integral (t = 24 h)
Glycine Betaine		42.85	5.53	4.94
Trigonelline		18.98	0	0
Dimethylthetin		21.06	15.57	10.04
DMSP		19.99	12.85	5.09
Selenobetaine		17.70	13.89	9.76
Arsenobetaine		45.77	0	0

## 6.6.3.2 Inhibition Studies

**Table 6.4** Results of the inhibition studies. Data was also recorded at  $t = 1$  h, but did not show any signs of inhibition, and so is not recorded below.

Betaine Name	Betaine Structure	Betaine Integral ( $t = 24$ )	DMG Integral ( $t = 24$ h)	Methionine Integral ( $t = 24$ h)	% Inhibition
Glycine Betaine		42.09	5.53	4.94	
Trigonelline		19.17	5.69	4.53	0
Dimethylthetin		19.75	Uncertain; DMT and DMG co- resonant	9.01	Competitive Inhibition
DMSP		18.03	Uncertain; DMSP and DMG co- resonant	4.60	Competitive Inhibition
Selenobetaine		10.03	0	17.05	100 Competitive Inhibition
Arsenobetaine		45.51	5.40	4.97	0

**Table 6.5** Comparison of rat and hagfish activity

Betaine Name	Betaine Structure	Rat demethylated product integral	Hagfish demethylated product Integral
Glycine Betaine		7.51	5.53
Trigonelline		0	0
Dimethylthetin		19.31	15.57
DMSP		16.74	12.85
Selenobetaine		10.97	13.89
Arsenobetaine		0	0

## 6.6 Discussion

Acetone powders can provide a convenient source of protein for studies of BHMT. The results presented here show that BHMT can be obtained from acetone extraction. Acetone powders are frequently stored for extended periods with little further degradation of proteins, so acetone powders may become the commercial source of choice for BHMT research. Several companies provide a range of mammalian liver acetone powders, as well as the snail and shark samples available from Sigma.

There are some reports of unstable BHMT extracts in the literature, notably a report by Lee *et al* report a rat-liver extract which was unstable, unless in the presence of homocysteine and DMG.<sup>31</sup> The rat-liver extracts which were obtained after anion-exchange chromatography and discussed in **Chapter 2** were also found to be unstable, although the recombinant enzyme which is reported by the Garrow research group is quite stable, and can be stored for some time without loss of activity.<sup>1,9,10,41</sup> While no activity was detected in butter-shark liver acetone powder, it is possible that the shark BHMT is unstable which prevents its detection in acetone powder.

Hagfish BHMT can be partially purified using a scheme similar to that used for rat liver. The resulting hagfish BHMT also has similar properties to rat BHMT, in that its activity is remarkably heat stable, withstanding a 90 °C heat shock for 2 min – a treatment which denatures most other proteins. This implies some degree of similarity chemical structure between the two proteins.

The size exclusion chromatography showed BHMT activity the hagfish in fractions 21-24, the same fractions which exhibit BHMT activity when rat liver extracts are analysed. This result implies that the hagfish BHMT quaternary structure (based on the assumption of a similar ~45 kDa subunit to the rat BHMT subunit) is comparable to rat BHMT. Similar percent purifications are seen between the rat extraction and the hagfish extraction [**Chapter 2**,



**Section 2.6.3]**, although these produce solutions which are much less pure than the rat liver purification.

*N*-terminal protein sequencing on the ~45 kDa band identified in the SDS-PAGE analysis of BHMT active hagfish extracts did not give any results, despite two attempts at sequencing, and an *in situ* acid/alcohol/heat cycle to attempt deacetylation of the *N*-terminal amino acid. Initially, the wrong band was excised and submitted for analysis, revealing the first experimental evidence for the 'Hematopoietic antimicrobial peptide-37 precursor' protein. This confirms that hagfish protein was efficiently transferred during the PVDF blot, and further work is required to isolate, and sequence the hagfish BHMT protein.

There is one predicted BHMT alternate transcription site known in mice, one of which is anecdotally known as 'mega-BHMT', because it would encode a protein of 52 kDa; substantially bigger than BHMT.<sup>41</sup> It is unlikely that we are observing the product of 'mega-BHMT' in the kinetic and characterisation studies because the band excised from the PVDF membrane is conclusively placed along side a 45 kDa marker (Ovalbumin, Sigma).

The hagfish BHMT activity appeared to have similar specificity for methyl donor as the rat BHMT [Tables 6.3, 6.4, 6.5], in that group VI heteroatom betaines are good substrates for both hagfish and rat BHMT, and the inclusion of an extra CH<sub>2</sub> in the thetin series appears to decrease activity. Furthermore, the betaines which are substrates also appear to have competitive inhibitor properties, implying that they are all reacting at a common active site. This implies that the mechanism of BHMT action in hagfish is similar to that of rat BHMT, and that the binding of betaines to rat BHMT is likely to be the same for the hagfish enzyme.

## 6.7 Conclusion

Agnathans were selected for comparison to mammals for several reasons. Ericson had briefly reported BHMT activity in lamprey livers in 1960, and hagfish are easily sourced from commercial fisheries, being common as an off-catch of normal fishing activity, and are normally rapidly discarded. They are the only existent members of our most distant vertebrate relatives, and the presence of BHMT in their livers makes them an attractive prospect for study.

The BHMT purification used here relies on two critical physical properties, heat stability, and mass, both of which offer clues about the structure of the tertiary and quaternary structure of the protein. Hagfish BHMT behaved largely in the same way as rat BHMT during heat treatment and the size exclusion chromatography, implying a similar structure.

The solutions obtained from the hagfish purification were of unsuitable purity to conduct mass spectrometry or tryptic digests to confirm the presence of a BHMT like protein. Furthermore, it is difficult to predict what masses are expected, given that there is no existing data on the hagfish BHMT protein sequence.

No *N*-terminal sequence was obtained for the hagfish BHMT subunit. The most likely reason for this is *N*-terminal acetylation of the first residue in the hagfish. Alanine is highly conserved in the first amino acid position of BHMT, and is a known site for acetylation. *In situ* attempts at un-blocking the sequence using an in-house acid-alcohol-heat procedure were not successful. Further work will need to be done to confirm that there is sufficient protein on the PVDF blot and that the *N*-terminal is blocked. *In situ* cyanogen bromide cleavage at methionine residues would (if a subunit similar to rat BHMT is present) cleave the protein into nine fragments, eight of which will not have a blocked *N*-terminal. It may also be possible to elute the 45 kDa band and determine internal protein sequences after appropriate cleavage, using preparative HPLC.

The substrate specificity work observed also implies that hagfish BHMT and rat BHMT have a similar betaine binding site, although it should be noted that further assays using a wider range of betaines, in a similar way to that performed in **Chapter 5**, would be required to conclusively probe betaine binding in hagfish BHMT. This work, however, is a good step towards the full characterisation of hagfish BHMT.

## References

- (1) Breksa, A. P., III; Garrow, T. A. *Biochemistry* **1999**, Recombinant human liver betaine-homocysteine S-methyltransferase: Identification of three cysteine residues critical for zinc binding., *38*, 13991-13998.
- (2) Peariso, K.; Zhou, Z. S.; Smith, A. E.; Matthews, R. G.; Penner-Hahn, J. E. *Biochemistry* **2001**, Characterization of the zinc sites in cobalamin-independent and cobalamin-dependent methionine synthase using zinc and selenium X-ray absorption spectroscopy,, *40*, 987-993.
- (3) Evans, J. C.; Huddler, D. P.; Jiracek, J.; Castro, C.; Millian, N. S.; Garrow, T. A.; Ludwig, M. L. *Structure* **2002**, Betaine-Homocysteine Methyltransferase Zinc in a Distorted Barrel, *10*, 1159-1171.
- (4) Evans, J. C.; Huddler, D. P.; Hilgers, M. T.; Romanchuk, G. L.; Matthews, R. G.; Ludwig, M. L. *Proceedings of the National Academy of Sciences U.S.A.* **2004**, Structures of the N-terminal domains of methionine synthase imply large domain motions during catalysis.
- (5) Sowden, M. P.; Collins, H. L.; Smith, H. C.; Garrow, T. A.; Sparks, J. D.; Sparks, C. E. *Biochemical Journal* **1999**, Apolipoprotein B mRNA and lipoprotein secretion are increased in McArdle RH-7777 cells by expression of betaine-homocysteine S-methyltransferase, *341*, 639-645.
- (6) Durell, J.; Anderson, D. G.; Cantoni, G. L. *Biochimica et Biophysica Acta* **1957**, Synthesis of methionine by enzymic transmethylation. I. Purification and properties of thetin homocysteine methylpherase, *26*, 270-282.
- (7) Ericson, L. E.; Williams, J. N., Jr.; Elvehjem, C. A. *Journal of Biological Chemistry* **1955**, Partially purified betaine-homocysteine transmethylase of liver, *212*, 537-544.
- (8) Ericson, L. E. *Acta Chemica Scandinavica* **1960**, Betaine-homocysteine methyltransferases. I. Distribution in nature, *14*, 2102-2112.
- (9) Garrow, T. A. *Journal of Biological Chemistry* **1996**, Purification, kinetic properties, and cDNA cloning of mammalian betaine-homocysteine methyltransferase, *271*, 22831-22838.
- (10) Millian, N. S.; Garrow, T. A. *Archives of Biochemistry and Biophysics* **1998**, Human betaine-homocysteine methyltransferase is a zinc metalloenzyme, *356*, 93-98.

- (11) Mudd, S. H.; Finkelstein, J. D.; Irreverre, F.; Laster, L. *Journal of Biological Chemistry* **1965**, Transsulfuration in mammals. Microassays and tissue distributions of three enzymes of the pathway, *240*, 4382-4392.
- (12) Rao, P. V.; Garrow, T. A.; John, F.; Garland, D.; Millian, N. S.; Zigler, J. S., Jr. *Journal of Biological Chemistry* **1998**, Betaine-homocysteine methyltransferase is a developmentally regulated enzyme crystallin in rhesus monkey lens, *273*, 30669-30674.
- (13) Sturman, J. A.; Gaull, G. E.; Niemann, W. H. *Journal of Neurochemistry* **1976**, Activities of some enzymes involved in homocysteine methylation in brain, liver and kidney of the developing Rhesus monkey, *27*, 425-431.
- (14) Balinska, M.; Paszewski, A. *Biochemical and Biophysical Research Communications* **1979**, Betaine-homocysteine methyltransferase in the fungus *Aspergillus nidulans*, *91*, 1095-1100.
- (15) White, R. F.; Kaplan, L.; Birnbaum, J. *Journal of Bacteriology* **1973**, Betaine-homocysteine transmethylase in *Pseudomonas denitrificans*, a vitamin B12 overproducer, *113*, 218-223.
- (16) Pichereau, V.; Pocard, J.-A.; Hamelin, J.; Blanco, C.; Bernard, T. *Applied and Environmental Microbiology* **1998**, Differential effects of dimethylsulfonylpropionate, dimethylsulfonylacetate, and other S-methylated compounds on the growth of *Sinorhizobium meliloti* at low and high osmolarities, *64*, 1420-1429.
- (17) Pichereau, V.; Cosquer, A.; Gaumont, A.-C.; Bernard, T. *Bioorganic & Medicinal Chemistry Letters* **1997**, Synthesis of trimethylated phosphonium and arsonium analogs of the osmoprotectant glycine betaine; contrasted biological activities in two bacterial species, *7*, 2893-2896.
- (18) McKeever, M. P.; Weir, D. G.; Molloy, A.; Scott, J. M. *Clinical Science* **1991**, Betaine-homocysteine methyltransferase: organ distribution in man, pig and rat and subcellular distribution in the rat, *81*, 551-556.
- (19) Delgado-Reyes, C. V.; Wallig, M. A.; Garrow, T. A. *Archives of Biochemistry and Biophysics* **2001**, Immunohistochemical detection of betaine-homocysteine S-methyltransferase in human, pig, and rat liver and kidney, *393*, 184-186.
- (20) Baburina, O. K.; Shevyakova, N. I. *Russian Journal of Plant Physiology (Translation of Fiziologiya Rastenii (Moscow))* **1995**, Betaines as methyl-group donors in alfalfa shoots exposed to saline media, *42*, 764-771.

- (21) Incharoensakdi, A.; Waditee, R. *Current Microbiology* **2000**, Degradation of glycinebetaine by betaine-homocysteine methyltransferase in *Aphanothece halophytica*: effect of salt downshock and starvation., *41*, 227-231.
- (22) Jenkins, R. O.; Ritchie, A. W.; Edmonds, J. S.; Goessler, W.; Molenat, N.; Kuehnelt, D.; Harrington, C. F.; Sutton, P. G. *Archives of Microbiology* **2003**, Bacterial degradation of arsenobetaine via dimethylarsinoylacetate, *180*, 142-150.
- (23) Nyysola, A.; Kerovu, J.; Kaukinen, P.; von Weymarn, N.; Reinikainen, T. *Journal of biological chemistry* **2000**, Extreme halophiles synthesize betaine from glycine by methylation, *275*, 22196-22201.
- (24) Kacprzak, M. M.; Lewandowska, I.; Matthews, R. G.; Paszewski, A. *Biochemical Journal* **2003**, Transcriptional regulation of methionine synthase by homocysteine and choline in *Aspergillus nidulans*, *376*, 517-524.
- (25) Maw, G. A. *Biochemical Journal* **1959**, Thetin-homocysteine transmethylase. The distribution of the enzyme, studied with the aid of trimethylsulfonium chloride as substrate, *72*, 602-608.
- (26) Sunden, S. L. F.; Renduchintala, M. S.; Park, E. I.; Miklasz, S. D.; Garrow, T. A. *Archives of Biochemistry and Biophysics* **1997**, Betaine-homocysteine methyltransferase expression in porcine and human tissues and chromosomal localization of the human gene, *345*, 171-174.
- (27) Xue, G. P.; Snoswell, A. M. *Comparative Biochemistry and Physiology, Part B: Biochemistry & Molecular Biology* **1986**, Developmental changes in the activities of enzymes related to methyl group metabolism in sheep tissues, *83B*, 115-120.
- (28) Nakajima, K. *Nippon Suisan Gakkaishi* **1993**, Dimethylthetin- and Betaine-homocysteine Methyltransferase Activities from Livers of Fish, Chicken, and Mammals, *59*, 1389-1393.
- (29) Ericson, L. E. *Acta Chemica Scandinavica* **1960**, Betaine-homocysteine methyltransferases. III. The methyl-donor specificity of the transferase isolated from pig liver, *14*, 2127-2134.
- (30) Lee, M. B.; Blunt, J. W.; Lever, M.; George, P. M. *Analytical Biochemistry* **2004**, A nuclear-magnetic-resonance-based assay for betaine-homocysteine methyltransferase activity, *330*, 199-205.

- (31) Lee, K. H.; Cava, M.; Amiri, P.; Ottoboni, T.; Lindquist, R. N. *Archives of Biochemistry and Biophysics* **1992**, Betaine:homocysteine methyltransferase from rat liver: purification and inhibition by a boronic acid substrate analog, *292*, 77-86.
- (32) Emmert, J. L.; Garrow, T. A.; Baker, D. H. *Journal of Nutrition* **1996**, Hepatic betaine-homocysteine methyltransferase activity in the chicken is influenced by dietary intake of sulfur amino acids, choline and betaine., *126*, 2050-2058.
- (33) Emmert, J. L.; Webel, D. M.; Biehl, R. R.; Griffiths, M. A.; Garrow, L. S.; Garrow, T. A.; Baker, D. H. *Journal of Animal Science* **1998**, Hepatic and renal betaine-homocysteine methyltransferase activity in pigs as affected by dietary intakes of sulfur amino acids, choline, and betaine, *76*, 606-610.
- (34) Duerre, J. A.; Miller, C. H. *Analytical Biochemistry* **1966**, Preparation of L-homocysteine from L-homocysteine thiolactone, *17*, 310-315.
- (35) Uzzell, T.; Stolzenberg, E. D.; Shinnar, A. E.; Zasloff, M. *Peptides* **2003**, Hagfish intestinal antimicrobial peptides are ancient cathelicidins, *24*, 1655-1667.
- (36) Francesconi, K. A.; Edmonds, J. S. *Advances in Inorganic Chemistry* **1997**, Arsenic and marine organisms, *44*, 147-189.
- (37) Fujihara, J.; Kunito, T.; Kubota, R.; Tanabe, S. *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology* **2003**, Arsenic accumulation in livers of pinnipeds, seabirds and sea turtles: subcellular distribution and interaction between arsenobetaine and glycine betaine, *136C*, 287-296.
- (38) Khokiattiwong, S.; Goessler, W.; Pedersen, S. N.; Cox, R.; Francesconi, K. A. *Applied Organometallic Chemistry* **2001**, Dimethylarsinoylacetate from microbial demethylation of arsenobetaine in seawater, *15*, 481-489.
- (39) Sakurai, T.; Fujiwara, K. *British Journal of Pharmacology* **2001**, Modulation of cell adhesion and viability of cultured murine bone marrow cells by arsenobetaine, a major organic arsenic compound in marine animals, *132*, 143-150.
- (40) Yoshida, K.; Kuroda, K.; Inoue, Y.; Chen, H.; Wanibuchi, H.; Fukushima, S.; Endo, G. *Applied Organometallic Chemistry* **2001**, Metabolites of arsenobetaine in rats: does decomposition of arsenobetaine occur in mammals?, *15*, 271-276.
- (41) Garrow, T. *Personal Communication*.
- (42) Wang, J.; Dudman, N. P. B.; Lynch, J.; Wilcken, D. E. L. *Clinica Chimica Acta* **1991**, Betaine:homocysteine methyltransferase-a new assay for the liver enzyme and its absence from human skin fibroblasts and peripheral blood lymphocytes, *204*, 239-249.

Chapter 7

## *In Vivo* Studies of Mammalian Betaine

Metabolism



## CHAPTER 7: *In Vivo* Studies of Mammalian Betaine Metabolism

### 7.1 Introduction

*In vitro* data on enzyme properties may not reflect what happens *in vivo*, where there is a high level of cellular organization. *In vitro*, all parameters are essentially under the control of the researcher, with temperature, pH, and the concentrations of the substrates being known and regulated. *In vivo* there is cellular and organ compartmentalisation, active transportation of betaines and the excretion of betaines which can all complicate measurement of BHMT activity. The effect of betaines on the whole organism must be investigated separately from the *in vitro* measurements of enzyme kinetics and substrate specificity.

NMR spectroscopy can provide a useful tool for the measurement of betaines and methylamines in biological fluids. The assays used for kinetic measurements in the earlier chapters of this thesis were adapted and applied to the study of betaine metabolism in four separate studies.

#### 7.1.1 Methylation *in Vivo*

Methyl group cycles are crucial to most organisms. It is estimated that up to 75% of all the SAM generated in the body is used to synthesise creatine, but there are many other reactions which are linked through SAM to homocysteine metabolism.<sup>1,2</sup> Notably, DNA is methylated within the cell to regulate the expression of many genes, forming an important epigenetic trigger which can be used to control the functions of the cell.<sup>1,3</sup>

As a consequence of each of these methylations SAM is produced, which is directly hydrolysed to homocysteine. The link between CVD and homocysteine is now well established

(discussed in **Chapter 1**). Additionally, increased homocysteine concentrations have flow-on effects on other pathways present in the organism.<sup>4</sup> Many of the methylation reactions use homocysteine, in the form of *S*-adenosyl homocysteine (SAH) as a feedback inhibitor.<sup>1</sup>

It is known that homocysteine levels rise after a methionine load in humans,<sup>5</sup> and also in rats.<sup>6</sup> A good example of how homocysteine levels can be affected by alterations to the environment is in patients with Parkinson's disease. Treatment with L-DOPA results in the drug being transported to the brain, where it is decarboxylated, relieving the deficiency of dopamine, and reducing the symptoms. Plasma homocysteine levels in these patients are commonly 50% higher than in untreated healthy controls.<sup>7</sup> It has been shown in rats that the homocysteine elevating effect of L-DOPA is accompanied by a lowering of SAM levels, and was a consequence of methylation of the L-DOPA via catechol-*O*-methyl transferase (COMT, E.C. 2.1.1.6).<sup>7</sup>

Stead *et al* showed that an increased dietary creatine intake decreases the plasma homocysteine presumably by reducing the demand for creatine synthesis,<sup>8</sup> and thus reducing the methylation flux via that pathway. Creatine biosynthesis is known to account for up to 75% of *S*-adenosyl methionine *in vivo* although this is the subject of debate.<sup>1,8</sup> Creatine synthesis is, in turn, also affected by a number of different factors. Phosphate levels in plasma, ATP concentrations within the cell, hormonal regulation (notably thyroid hormones, although hydrocortisone up regulates creatine synthesis) and plasma pH have all been shown to be regulating factors in creatine synthesis, and might therefore have an influence on the homocysteine flux in the whole animal. While these factors are influencing creatine synthesis, they may not have an effect on homocysteine levels within the plasma (methionine synthase, BHMT and cystathionine  $\beta$ -synthase may remove homocysteine as fast as it is made) it would alter the demands placed on the various enzymes in the methionine-homocysteine pathway and, therefore, on betaine as a methyl donor.

### 7.1.2 Whole Animal Studies

Given the complications *in vivo* discussed in **Section 7.1.1**, it was important to look at how the betaines studied in this thesis affect the animal as a whole. The ultimate aim of these four projects was to determine the suitability of betaines for use as therapeutic agents for the lowering of homocysteine in subjects with hyperhomocysteinemia.

Glycine betaine has been used for many years to reduce high plasma homocysteine levels.<sup>9,10</sup> While it is effective against extremely high homocysteine concentrations (for example in cystathionine  $\beta$ -synthase or vitamin B<sub>6</sub> deficiency), it does not have much effect against lower homocysteine concentrations resulting from errors involving defects in methionine synthase.<sup>11</sup> Olthof *et al* have shown that daily betaine supplementation as low as 1.5 g is effective at lowering homocysteine in a post-methionine load situation (a test where the subjects are given a load of methionine which is converted into homocysteine).<sup>9</sup>

There are three reasons why these betaine treatments may not be as successful as hoped. Firstly, the *BHMT* gene is regulated strongly by factors such as betaine and methionine levels. Secondly, as BHMT produces DMG, a feedback inhibitor of BHMT, and so regulates its own activity. Thirdly, as methionine is reformed by the action of BHMT, it is likely to simply re-enter the SAM cycle and regenerate homocysteine.<sup>12</sup>

There is little that can be done about the first of these factors. The control exerted by methionine on *BHMT* is at the genetic level (see **Chapter 1**).<sup>13-15</sup> It is unlikely that there would be a simple way of altering the regulation these molecules have on the BHMT protein. The third factor is also, unfortunately, not possible to adjust. Diversion of plasma homocysteine out of the SAM-SAH cycle is not easily achieved. Methionine must be produced by the action of BHMT, and so, will logically re-enter the SAM-SAH cycle. However, each time that cycle is returned, 50% of the homocysteine will enter the CBS pathway and be removed permanently.<sup>16</sup>

It is possible that the feedback inhibition of BHMT by DMG is externally controllable. BHMT is able to process methyl groups from a number of different substrates, notably dimethylthetin (DMT) and dimethylsulfoniopropionate (DMSP). Both of these sulfur analogues of glycine betaine are more rapidly used by BHMT to convert homocysteine to methionine, but produce demethylated products which will then further inhibit the enzyme. The utilization of these thetins should increase BHMT's capacity to remove homocysteine.

It is likely that the thetins will also, however, interact with the other facets of betaine homeostasis and transport. In order to investigate the effect these betaines have on the homocysteine system, including some of the betaines not known to interact with BHMT, four experiments were proposed and carried out.

The experiments which make up this chapter were all done in collaboration with other researchers in the betaine research group at Canterbury Health Laboratories. Animal work was largely conducted by Dr Sandy Slow and the sheep study also involved Madhusudan Vasudevamurthy. The DMG and coffee studies were also largely conducted by Dr Sandy Slow, with the assistance of Wendy Miller.

All studies conducted here used  $^1\text{H}$ -NMR spectroscopy to locate and quantify a betaine as a product of the *in vivo* study – normally in the urine. The methods developed in earlier chapters of this thesis were applied and I have been involved in the planning, methodology and execution of the analyses involving NMR spectroscopy presented.

## 7.2 Rat Models of Betaine Metabolism

### 7.2.1 Betaine Homeostasis

Glycine betaine therapy is successful in lowering plasma homocysteine levels in homocystinuria, and also in patients with chronic renal failure,<sup>17,18</sup> it is likely that betaines are capable of using BHMT to lower homocysteine. There are a number of betaines which are known to interact with BHMT, notably DMT and DMSP, but proline betaine (PB) and trigonelline could also potentially act as substrates and inhibitors of BHMT and affect homocysteine metabolism. At the time of this study, porcine BHMT had been shown to have low activity with proline betaine, about 20% of the activity of glycine betaine.<sup>19</sup> *In vivo* this could lead to a competitive inhibition of BHMT or assisting glycine betaine to lower homocysteine. Trigonelline is known to be transported by the same betaine transporter proteins as glycine betaine and also potentially interact with the active site of BHMT.<sup>20</sup> BHMT has shown activity with DMT and DMSP, with relative  $K_m$  of 30 and 7 times those of glycine betaine, so they would be expected to assist in the removal of homocysteine.<sup>21</sup>

### 7.2.2 Study design

Six groups of 8 inbred Lewis rats were given a low betaine diet for a period of one week prior to the application of experimental procedures. In five of the groups of 8 animals, six animals were given a betaine subcutaneous injection and the remaining two animals acted as controls. Additionally, one group were injected with isotonic 5% glucose solution. Each betaine injection contained 1.5 mM of the betaine (one of glycine betaine, DMG, proline betaine, trigonelline, DMT or DMSP) and the control animals received a NaCl treatment of the same volume. The injections were hypertonic at 500 mosmols/L.

Injections were chosen to prevent uncertainties that may have been introduced if the betaines were administered via the food supply, providing less stress to the animals than gastric tube administration. Also, microbial metabolism of the betaines in the digestive tract was avoided.

The rats were housed individually in metabolism cages to collect urine for 48 hours. Urine was collected for the 0-8 hours and 8-24 hours pre-treatment, then the injections given, and another set of urine for the 0-8 hours and 8-24 hours post treatment. The rats were given their respective treatments twice, with a week's interval between them. On the second treatment day, the rats had 200  $\mu$ L blood samples taken at 0, 0.5, 2, 4, 8 hours respectively, and finally, a 24 hour sample was taken after sacrifice of the rat via cardiac puncture.

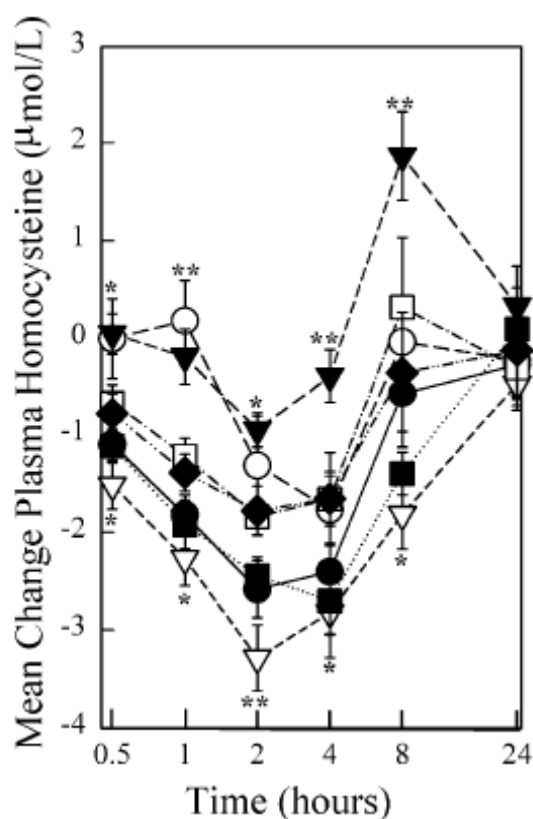
The blood samples were all analyzed for plasma homocysteine concentration (separated and stored at -20 °C). Betaine analysis for glycine betaine, DMG, proline betaine, trigonelline, DMT and DMSP in both the plasma and the urine was conducted using the HPLC method described in **Chapter 1**.<sup>22</sup>

<sup>1</sup>H-NMR spectroscopy was used to identify the expected demethylated products of the BHMT reaction (DMT and DMSP). The methods were the same as those described in **Chapter 3**. Metabolites were quantified by reference to an internal acetonitrile spike of 5 mM which also served as a chemical shift reference at 1.9 ppm. Standard lines for the demethylated products (methylthioacetate and methylthiopropionate) as well as the betaines, were prepared over six points (0.05, 0.1, 0.5, 1, 3, 5 mM), and the ratios of the peak integrals to the acetonitrile were calculated.

### 7.2.3 Results

Plasma homocysteine results were expressed as an increase or decrease compared to baseline levels, as determined for the first ( $t = 0$  hrs) blood sample. The mean baseline homocysteine for the 48 rats was  $8.5 \pm 0.2$   $\mu$ M (four rats did not give data). All rats experienced an initial drop in plasma homocysteine over the first 4 hrs, maybe a consequence of stress which is known to cause a rapid drop in plasma homocysteine.<sup>23</sup> The plasma homocysteine concentration between the two control groups (saline and 5% glucose) was not statistically different. Proline

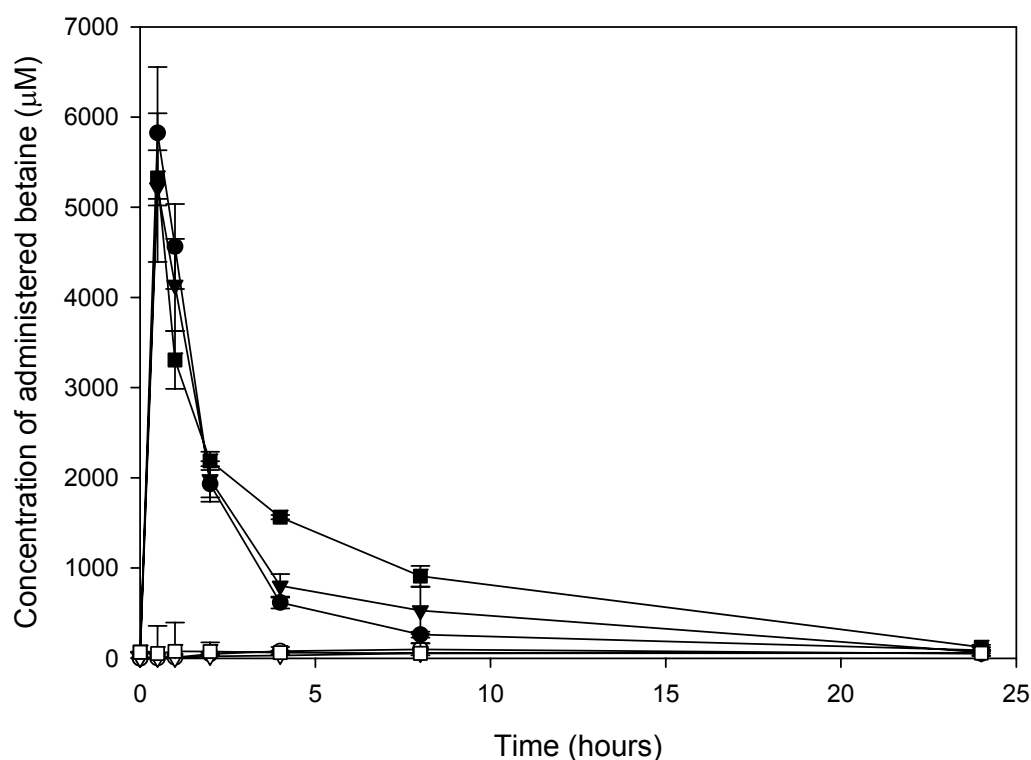
betaine and trigonelline both caused an increase in plasma homocysteine (the effect of proline betaine was still significant after 8 hours) while glycine betaine, DMT and DMSP all caused homocysteine to drop [Figure 7.1].



**Figure 7.1** Plasma homocysteine after betaine treatment. Data is expressed as mean change ( $\mu\text{M/L}$ )  $\pm$  S.E.M.; glycine betaine (●); proline betaine (▼); trigonelline (○); DMSP (■); dimethylthetin (▽); glucose (□); controls (◆). \*\*Highly significant difference compared to controls,  $P = 0.01$ . \*Significant difference compared to controls  $P = 0.05$ .

The plasma samples were also analyzed for glycine betaine – evidence that the injected betaines were interacting with betaine transport, or inhibiting metabolism of glycine betaine by competitively inhibiting BHMT. Plasma from the rats given glycine betaine were also analysed for DMG to see if there was increased methyl transfer as a consequence of increased betaine levels, and the decrease in homocysteine.

It was clear that the betaines were rapidly absorbed, with maximum plasma concentrations being achieved after 30 min. DMT achieved a maximum concentration at  $8.9 \pm 0.5$  mM, and the other betaines were also equilibrated after 30 min achieving concentrations of  $7.7 \pm 0.7$  mM (DMSP),  $5.8 \pm 0.6$  mM (proline betaine),  $5.4 \pm 0.3$  mM (glycine betaine), and  $5.0 \pm 0.2$  mM (trigonelline).



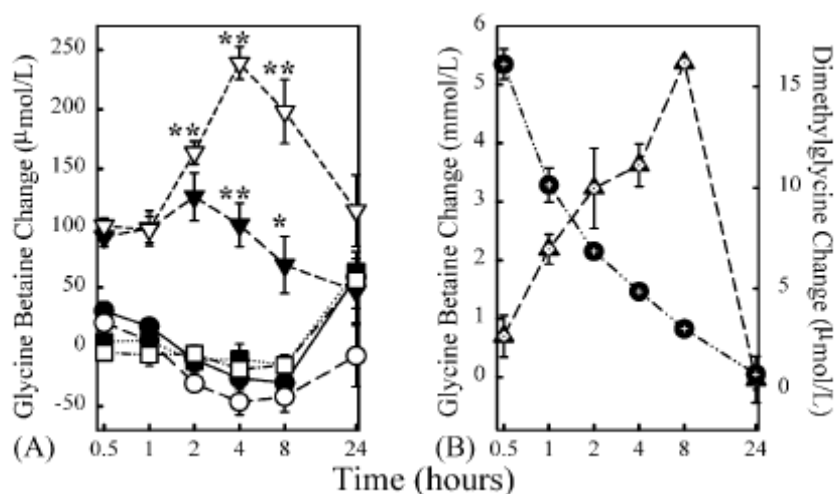
**Figure 7.2** Plasma concentrations of the administered betaines, showing the rapid absorption and equilibration of the betaines into the plasma. All samples had achieved their peak plasma concentration within 30 min of administration. Proline betaine (●), Proline betaine controls (○), trigonelline (▼), trigonelline controls (▽), glycine betaine (■), glycine betaine controls (□).

The control rats, and the glucose injection group had no significant difference in plasma glycine betaine concentration, and the trigonelline and proline betaine injections also had no significant effect on the plasma glycine betaine concentration [Figure 7.1 (A)]. DMT and DMSP both caused a significant increase in plasma glycine betaine levels, which was highly significant at 2, 4, and 8 hours after injection of DMT.



Given that glycine betaine can be demethylated by BHMT to form DMG, it followed that after injection of glycine betaine, there was an initial rise in plasma glycine betaine (peaking after 30 min) which decayed exponentially to baseline over the 24 hour period. This was accompanied by an increase in plasma DMG concentration, which was observed over the first 8 hours, but had returned essentially to baseline after 24 hours [Figure 7.3 (B)].

DMT significantly decreased the concentration of DMG in the plasma between one and 8 hours following the injection. At the point of greatest difference (four hours after injection) this was  $4.5 \pm 1.2 \mu\text{M}$  ( $p < 0.001$ ). This was contrasted by an increase in DMG between 8 and 24 hours following the injection. Methylthioacetate (MTA) was observed by HPLC analysis in the plasma of the DMT treated rats, and reached a maximum of  $230 \pm 24 \mu\text{M}$  at 8 hours after injection. It is important to note that MTA is not known to occur naturally, and can only have been produced by demethylation of DMT. Furthermore, the peak assigned to MTA was not observed in samples from baseline samples, or from any other rat urines.



**Figure 7.3** Changes in plasma glycine betaine and DMG after injection of betaine during the rat study. (A), the mean change ( $\mu\text{M/L}$ )  $\pm$ S.E.M in plasma glycine betaine after injection of proline betaine (●); trigonelline (○); DMSP (▼); dimethylthetin (▽); glucose (■); controls (□). Initial concentrations are: proline betaine (104  $\mu\text{M}$ ), trigonelline (102  $\mu\text{M}$ ), DMSP (99  $\mu\text{M}$ ), DMT (106  $\mu\text{M}$ ), glucose (78  $\mu\text{M}$ ), control (88  $\mu\text{M}$ ) (B) Mean change ( $\mu\text{M/L}$ )  $\pm$ S.E.M in plasma glycine betaine (●, initial concentration = 69  $\mu\text{M}$ ) and DMG (▲, initial concentration = 69  $\mu\text{M}$ ). \*\*Highly significant difference compared to controls,  $P = 0.01$ . \*Significant difference compared to controls  $P = 0.05$ .

Each treated rat received 1.5 mM of betaine following which  $41 \pm 3.5\%$  of the glycine betaine,  $72 \pm 8.7\%$  proline betaine,  $79 \pm 5.1\%$  trigonelline,  $66 \pm 2.5\%$  DMSP and  $7.7 \pm 0.9\%$  DMT was excreted into the urine after 24 hours. Glycine betaine excretion was elevated in the rats which received betaine injections and saline injections, but not in the glucose control group.

After injection with DMT and DMSP, glycine betaine excretion was significantly elevated for the first 8 hours, and significantly lowered for the 8-24 hour period post injection. NMR analysis was used to investigate the urine of the DMT and DMSP treated rats as HPLC could not accurately identify the demethylated products of these two metabolites, methylthioacetate (MTA) and methylthiopropionate (MTP) respectively.

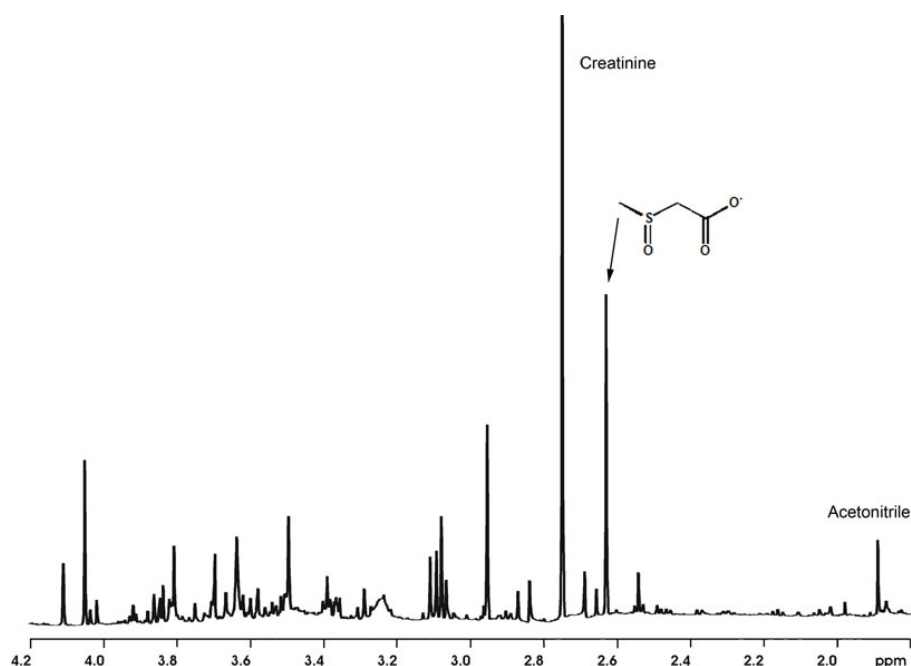
MTA was identified as a singlet appearing at 1.97 ppm in the urine NMR spectrum, and verified by spiking of the samples. Using the NMR methods described in Chapter 3, it was

quantified as  $0.41 \pm 0.03$   $\mu\text{moles}$  and  $0.57 \pm 0.08$   $\mu\text{moles}$  for the 0-8 hour and 8-24 hour periods respectively post-injection. As this represents  $<0.1\%$  of the 1.5 mmole DMT injection, it was hypothesised that the MTA was being further metabolised, possibly by oxidation to the sulfoxide.

A sample of the sulfoxide of MTA was prepared by addition of one molar equivalent of 30%  $\text{H}_2\text{O}_2$  to a sample of MTA in methanol. The sulfoxide of MTA has a pair of enantiotopic protons, giving rise to a unique set of doublets which were easily recognisable as evidence of the sulfoxide in rat urine [Figure 7.4].

The presence of this compound was then confirmed in the urine of the DMT treated rats, and quantified at  $138.4 \pm 15.8$   $\mu\text{mole}$  between 0-8 hours and  $33 \pm 3.2$   $\mu\text{mole}$  between 8-24 hours. The sulfoxide of MTA decarboxylates to give dimethylsulfoxide (DMSO) and carbon dioxide over time, and so these figures are likely to be a minimum excretion estimate. Despite confirming the presence of DMSO in aqueous standards of the sulfoxide, there was no observed DMSO in the rat urine. The sulfoxide of MTA accounted for a further 11% of the DMT injection.

There was no observed MTP or *N*-methyl proline in the DMSP or proline betaine treatment groups.



**Figure 7.4**  $^1\text{H}$ -NMR spectrum of acidified rat urine following DMT treatment. The presence of the methylthioacetate sulfoxide is clearly seen at 2.63 ppm.

#### 7.2.4 Discussion

It is clear that glycine betaine, DMT and DMSP are all capable of lowering circulating levels of homocysteine in a whole animal model. Although all three compounds are known to be substrates of BHMT, the circulating concentration of homocysteine did not drop more than 30% of the original baseline levels.

It is thought that this may have been a consequence of the futile methylation cycle mentioned in **Section 7.1.2**. After homocysteine is remethylated by BHMT, it is either incorporated into proteins as the protein-amino acid, or it is converted into SAM, to be used in another methylation reaction.<sup>12</sup>

The glycine betaine treated rats may have also experienced a significant suppression of BHMT activity, as a consequence of the DMG being produced. It is, however, impossible to dissect

that information out of the data collected. The significant portion of injected glycine betaine which was recovered from the urine may also be evidence that the glycine betaine was not being utilised and was being excreted.

The absence of MTP, the demethylated product of the DMSP reaction, does not in itself exclude the possibility that it was produced. The sulfoxide of this compound would be similarly unstable, and could decarboxylate as the MTA sulfoxide did. The slight increase in plasma glycine betaine and the lower homocysteine concentration imply that the DMSP was being metabolised by BHMT. It is clear from the presence of MTA and its sulfoxide in the urine, however, that DMT was being demethylated, and is responsible for the significant drop in plasma homocysteine, and the increase in glycine betaine excretion.

In contrast, trigonelline and proline betaine were not demethylated and were excreted unchanged in the urine. Notably, all the betaines gave an increase in glycine betaine excretion, consistent with the findings of Lever *et al*/ who showed that proline betaine increased glycine betaine in the urine, possibly by blocking transportation in the proximal tubules of the kidney.<sup>24</sup> It is not unreasonable to hypothesise that the same mechanism could account for the elevated excretion in the rats.

NMR spectroscopy was a rapid and simple method for the verification of the two unknown compounds in rat urine (methylthioacetate and its sulfoxide). Each measurement was complete, offering the identification and quantification of each unknown within 2 min.

## 7.3 Chronic Renal Failure in Sheep

### 7.3.1 Renal Failure and Homocysteine

Human patients who are suffering chronic renal failure (CRF) experience a dramatic increase in plasma homocysteine level.<sup>25</sup> Moreover, they can also experience 10-20 times the incidence of CVD compared with normal people. The mechanism responsible for this increase in CVD in CRF is unknown, but glycine betaine depletion may play a part in the condition. During CRF, glycine betaine is cleared at an elevated rate by the kidneys, giving a lower concentration in the plasma, and if left without replacement, this may lead to a chronic increase in plasma homocysteine, and therefore, an increased risk of CVD.

A model of CRF was set up by Dr Sandy Slow to investigate the addition of DMT to dialysis fluid being used in Continuous Ambulatory Peritoneal Dialysis (CAPD). DMT was chosen for its efficient methyl transfer in BHMT, which should result in the greatest drop in homocysteine. The analysis was conducted in a similar way to the rat study [Section 7.2], with most betaine analyses being conducted by HPLC, and <sup>1</sup>H-NMR spectroscopy being used to analyse betaines that could not be easily be discerned by HPLC.

### 7.3.2 Study Design

Four Coopworth sheep (wether hoggets) weighing between 36 kg and 52.5 kg were housed in metabolism cages, fed a diet consisting of lucerne chaff, crushed barley and all-purpose ruminant pellets, and were given 4 L of tap water each day. The animals were adjusted to this for 1-2 weeks before experimental procedures were initiated.

A standard (47 cm universal) double-cuff Tenckhoff peritoneal dialysis silicone rubber catheter was inserted into the peritoneum of each animal using appropriate analgesia and anaesthetic and the surgery site was allowed to heal for up to two weeks. The catheters were flushed daily with 1 L of 1.36% glucose solution and were treated with concentrated heparin

(10,000 units) and corticosteroid (Depo-medrol<sup>®</sup>, 20 mg prednisolone) in a 10 mL volume to ensure they were not blocked.

Following healing of the catheter insertion site a full bilateral nephrectomy was conducted using appropriate analgesia and anaesthetic on three animals, to generate acute renal failure. Water was restricted to 1 L/day for the nephrectomised animals. Peritoneal dialysis commenced within 2 hours of recovery from nephrectomy surgery using a standard human dialysis regime consisting of 4 exchanges per 24 hours using either 1.5 L or 2 L of dialysis fluid per exchange and was employed for up to 14 days.

Blood samples (5 mL) were collected daily from each animal via jugular venipuncture into lithium heparin vacutainer tubes. An aliquot of whole blood was removed for haematocrit measurement and plasma was separated from the remaining blood by centrifugation at 2000 g for 10 min. Heart rate, respiration rate, rectal temperature, food and water intake and behaviour of all animals were monitored daily, both pre and post nephrectomy.

Plasma concentrations of electrolytes (sodium, chloride, potassium, calcium, magnesium and phosphate), creatinine, glucose, albumin, bilirubin, urea and urate were measured daily for all animals both pre and post nephrectomy.

DMT was added to the dialysis fluid at 10 g per exchange except in the case of one animal which received 5 g per exchange. Dialysis fluid samples were assayed for methylthioacetate (the expected demethylated product of DMT) and methylthioacetate sulfoxide by <sup>1</sup>H-NMR as previously described [Section 7.2.2].

### 7.3.3 Results

The first two nephrectomised animals which were treated with DMT died within 48 hours of exposure to DMT. Attention was then turned to two other subjects, one of which had been nephrectomised but had no exposure to DMT and another which had intact kidneys and was

placed onto dialysis. After exchanges with DMT were given, both showed rapid signs of DMT toxicity. The conclusion was that DMT was toxic regardless of dosage (the non-nephrectomized sheep received 5 g DMT per exchange, compared to 10 g per exchange for the other animals), and kidney function. All sheep died within 48 hours of exposure, although the onset of symptoms was subject to individual variation.

DMT toxicity resulted in a high heart rate, laboured breathing, salivation and lethargy. Animals became non-responsive, lost appetite and stopped drinking. One animal was withdrawn from DMT treatment after initial signs of respiratory distress, but still continued to decline until euthanized.

Plasma concentrations of DMT at death ranged from 0.5-7.8 mM, and were undetectable before exposure to DMT. The expected demethylated product, methylthioacetate, and its sulfoxide were not detected in plasma or dialysis fluid by NMR spectroscopy, or by HPLC.

Post mortem analysis of the DMT treated sheep revealed significant differences in the lungs. The DMT caused a consolidation of the lungs, resulting in an abnormal red-purple colouration and the lungs sank when placed in water. It was concluded that the sheep had died from lung failure. The lung was examined histologically, and it was concluded that the damage was consistent with Diffuse Alveolar Damage (DAD), consisting of hyaline membranes and proteinaceous exudates. When exhibited by adult patients, the condition is known as Adult Respiratory Distress Syndrome (ARDS).

#### 7.3.4 Discussion

Given the success of DMT in the rat study [Section 7.2], the toxicity of DMT in sheep was unexpected. The rats showed no signs of adverse reaction, and experienced rapid drops in plasma homocysteine. More importantly, two sheep in a pilot study which were given acute sub-cutaneous injections of 10 g of DMT showed no effects of DMT toxicity.



There are several potential explanations for the differences in metabolism observed. Firstly, sheep may have a species-specific response to DMT. They certainly have differences in BHMT distribution: rats express BHMT in the liver and have extremely low activity in the kidney<sup>26-28</sup>, sheep express BHMT in the pancreas to levels which exceed those in the liver, as well as having significant activity in the kidney.<sup>29</sup> This may lead to differences in DMT metabolism between the species.

In rats the demethylated product (methylthioacetate) was observed as well as the sulfoxide of methylthioacetate. Both compounds were absent from the sheep samples, indicating that there may be a difference in metabolism between them. It should also be noted that sheep have a rumen, containing microbes which may take DMT and alter its metabolism significantly, producing toxic chemicals which led to the lung failure observed.

The rats were exposed to only two doses of DMT at approximately 460 mg/kg, whereas the sheep were receiving approximately 500-800 mg/kg DMT daily, over four exchanges. While the rapid failure of the lungs in sheep indicates that the onset of toxicity was rapid, the constant exposure to relatively high levels of DMT, in addition to any metabolic differences, may lead to a greater effect than that seen in the rats. In rats, the DMT concentrations after 24 hours were 20-fold lower than those observed in the sheep, (0.5-7.8 mM), showing that the exposure in rats may have been insufficient to show toxic effects.

Finally, while unlikely, the route of administration was different for the animals which experienced DMT toxicity, indicating that route may be significant for toxicity. The rapid absorption and distribution of thetins, however, would indicate that this is unlikely to be a significant factor.

As with the rat metabolism study, NMR spectroscopy was a rapid method for the detection and quantification of DMT and its demethylated product MTA. Measurements were conducted on urine or dialysis fluid and were obtained much faster than by HPLC (2 min for NMR spectroscopy, versus 45-60 min for HPLC).

## 7.4 Coffee and Trigonelline

### 7.4.1 Coffee Consumption and Homocysteine

It has been shown that coffee consumption is correlated with plasma homocysteine levels.<sup>30,31</sup> This correlation appears to be independent of the preparation of the coffee, with filtered and unfiltered coffee both increasing plasma homocysteine by as much as 10%.<sup>32,33</sup> A total abstention from coffee leads to a reduction in total homocysteine.<sup>34</sup> It is known that caffeine interacts with homocysteine concentration, but there are other components of coffee which are glycine betaine analogues, and may affect BHMT *in vivo*.

Trigonelline is a naturally occurring betaine found in high concentrations in coffee up to 1% of the dry material in roasted coffee beans. At the time of the study, the effects of trigonelline on BHMT extracts were not known, and it was suspected from the rat study [Section 7.2] that trigonelline may have a homocysteine raising effect *in vivo*. It is important to note that this homocysteine elevating effect could be caused directly through interactions with BHMT, or through interactions in betaine transportation.

### 7.4.2 Study Design

After approval from the Canterbury Medical Ethics Committee, 8 males (18-38 years) were recruited and were tested for normal homocysteine, folate, vitamin B<sub>12</sub> and haemoglobin concentration. None of the subjects were taking B vitamin supplements, all were non-smokers and had no abnormal medical history.

All were judged to be healthy after a physical examination. Latin-square design was used to randomise the three treatments consisting of 100 mL water, 50 mg trigonelline in 100 mL water, and 5 g instant coffee (containing ~50 mg trigonelline) dissolved in 100 mL water. Each treatment was separated by a one week wash-out period. Subjects were asked to abstain from foods which

were known to be high in trigonelline, including coffee, for 48 hours prior to the study days,<sup>35</sup> and they fasted for 12 hours prior to study commencement.

Indwelling catheters were inserted, and fasting base-line blood samples (10 mL) were withdrawn into EDTA vacutainers. After treatment, blood samples were withdrawn every hour, and urine samples were collected every second hour for 8 hours. After each blood sample, subjects were given 150 mL water. After 8 hours, subjects collected an 8-24 hour urine sample overnight, and returned the next morning for a final blood sample. Standardised breakfasts and lunches were prepared from foods known to be low in glycine betaine and trigonelline.<sup>35</sup>

Plasma was prepared by centrifugation of the blood samples at 2000 g for 10 min. Urine volume was recorded, and a 10 mL aliquot was removed and frozen at -20 °C until required for analysis. Samples were analysed using standard Canterbury Health Laboratories clinical tests, with the exception of DMG and trigonelline, which were measured using the <sup>1</sup>H-NMR spectroscopy assay described in **Chapter 3** of this thesis.

The effect of the treatments on plasma homocysteine was expressed as a function of difference compared with baseline levels.

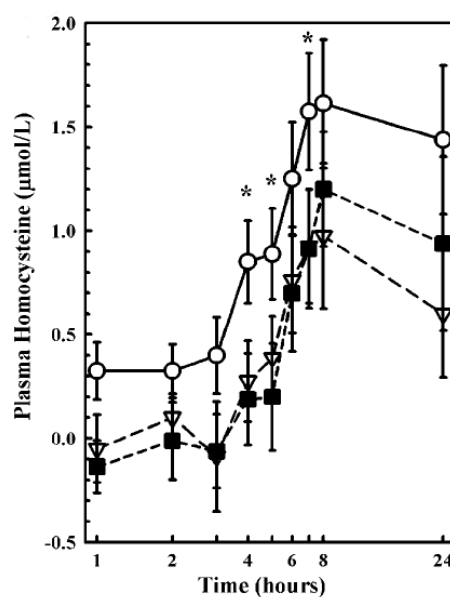
### 7.4.3 Results

Mean baseline plasma homocysteine concentrations for days 1, 2 and 3 were  $8.0 \pm 0.2$ ,  $7.8 \pm 0.2$  and  $7.8 \pm 0.2$   $\mu$ M respectively. Plasma homocysteine was significantly elevated after coffee consumption compared with water and trigonelline between one and 8 hours after treatment ( $P = 0.019$ , **Figure 7.5**). Water and trigonelline did not differ significantly during the study ( $P = 0.789$ ). The mean difference in plasma homocysteine was significantly elevated for coffee consumption at 4 hours ( $P = 0.0006$ ), 5 hours ( $P = 0.0013$ ) and 7 hours ( $P = 0.0024$ ).

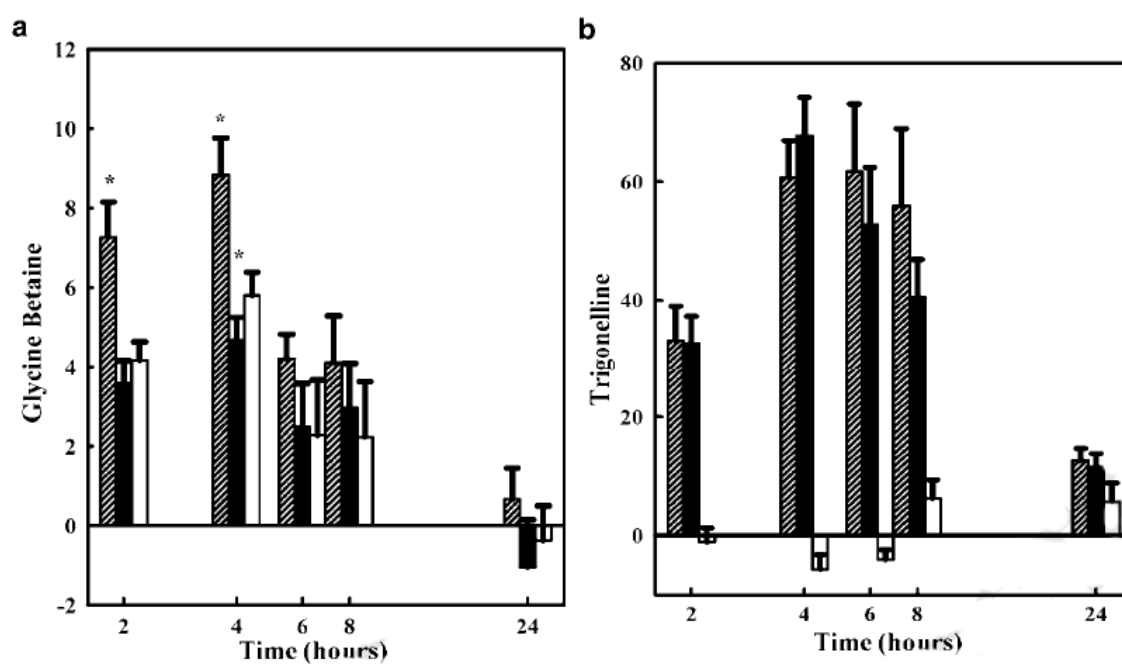
Mean baseline plasma glycine betaine was  $41.2 \pm 4.4$ ,  $41.9 \pm 3.7$  and  $43.1 \pm 5.7$  mM over the three study days respectively. It was significantly elevated at 1 hour following trigonelline,

when compared with the water and coffee consumption ( $P = 0.0035$  and  $0.0040$  respectively). This effect had gone after 2 hours. There was no significant difference between coffee and water for plasma glycine betaine.

Baseline glycine betaine excretions were measured as ratios to creatinine [Figure 7.6]. They were  $3.8 \pm 0.7$ ,  $7.1 \pm 1.0$  and  $5.0 \pm 0.7$  mmol/mol creatinine. Coffee consumption lead to an increase in glycine betaine excretion at two hours by  $7.3 \pm 0.9$  mmol/mol creatinine and by  $8.8 \pm 0.9$  mmol/mol creatinine at four hours. This was significantly elevated when compared to trigonelline and water. Trigonelline lead to a lowered excretion of urine glycine betaine at four hours which was significant, although only at that time point. Trigonelline excretion in the urine was clearly elevated post-trigonelline and coffee consumption, and these two treatments did not have any significant differences over any of the time points. The average baseline trigonelline concentrations were  $6.5 \pm 1.8$ ,  $5.2 \pm 0.8$  and  $6.0 \pm 0.8$  mmol/mol creatinine for the study days respectively.



**Figure 7.5** Plasma homocysteine as a function of time over the three treatments. Coffee (○) was significantly different (\*) at the 4, 5 and 7 hour time point compared to trigonelline (▽) and water (■).



**Figure 7.6** Urine excretion of glycine betaine, and trigonelline following Coffee (▨), trigonelline (■) and water (□). All y-axis values are in units of mmol/mol creatinine.

#### 7.4.4 Discussion

Coffee consumption gave rise to rapid increases in plasma homocysteine concentration by approximately 10%. Given the previous studies which indicated that chronic coffee consumption led to a higher plasma homocysteine,<sup>30,32,33</sup> this result is important, confirming those results and showing the effect acutely.

The trigonelline treatment did not result in any significant increased homocysteine concentration in the plasma, indicating that it is unlikely to be the active compound giving the results noted in the chronic consumption studies.<sup>30,32,33</sup> No direct evidence for the inhibition of BHMT activity *in vivo* could be obtained by this study, although the elevated glycine betaine excretion at two and four hour time points, combined with lowered plasma glycine betaine concentrations, indicate that coffee consumption may have an effect on the transportation and homeostasis of glycine betaine. Curiously, trigonelline alone did not have this effect, showing that there may be some other active compound in coffee leading to this observation.

There was an increase in plasma homocysteine after the consumption of coffee, which may have been caused by the inhibition of BHMT activity. If coffee consumption lowers the glycine betaine pool available to BHMT (by increasing the excretion of betaine) then the observed increase in plasma homocysteine may be explained.

Roughly 50% of the increase in homocysteine was attributed to caffeine from the coffee<sup>36</sup> and chlorogenic acid, also present in high levels in coffee, is known to increase homocysteine.<sup>37</sup> The exact reason for the increase in homocysteine is likely to be complex, and involve a number of the compounds present in the coffee. This experiment has excluded trigonelline from the likely list of active compounds which can increase homocysteine.

NMR spectroscopy showed it ability to identify and quantify methylamine compounds with no chemical manipulation and with rapid turnover. There was no clinical assay for trigonelline available at Canterbury Health Laboratories, and NMR spectroscopy was able to obtain results with

a 2 minute turnover of samples. DMG measurements by HPLC are complicated by co-elution with other compounds. NMR spectroscopy was employed to quantify the urine excretion in the subjects, as the DMG methyl-singlet signal is clear and separate from other peaks in the spectrum.

## 7.5 Dimethylglycine (DMG) Supplementation

### 7.5.1 Dimethylglycine and Methylation

Like glycine betaine and choline, DMG is an important source of methyl groups, which can be catabolised and used to form folate. However, the role of DMG in the regulation of plasma homocysteine is not clear. DMG is a potent feedback inhibitor of BHMT activity<sup>38</sup> and BHMT makes up as much as 50% of the remethylation of homocysteine to methionine in the body.<sup>16</sup> These interactions would lead to the prediction that high DMG would lead to a high homocysteine concentration.

DMG, however, can donate both of its remaining methyl groups to form 5'-methyl-tetrahydrofolate (mTHF). The two equivalents of mTHF formed from the metabolism of DMG can then go on via methionine synthase to remove homocysteine. Methionine synthase is responsible for the remaining 50% of the homocysteine which is recycled back to methionine.<sup>16</sup> This information leads to the assumption that high DMG decreases plasma homocysteine: the opposite prediction to the interaction with BHMT.

Choline metabolism leads to the production of DMG in most mammalian cells, however, DMG can be purchased over the counter in health food stores (known as 'pangamic acid', or 'vitamin B<sub>15</sub>') as a supplement for the enhancement of athletic abilities, improvement of epileptic symptoms, alleviation of autism and the stimulation of the immune system.<sup>39</sup>

There have been a few clinical studies of DMG supplementation, and none have any evidence to support most of the claims made by the health food industry. None of the studies performed to date, however, have looked specifically at the effect of DMG on homocysteine metabolism. Given the potential for DMG to effect plasma homocysteine, a study was designed to investigate the interaction of DMG in patients with Chronic Renal Failure (CRF). CRF patients have



elevated plasma homocysteine levels and should show any effects of DMG supplementation more readily than the normal population.

### 7.5.2 Study Design

After approval from the Canterbury Medical Ethics Committee, 8 pre-dialysis male CRF patients (44-75 years) were recruited from the Christchurch Hospital Nephrology Database. One of the patients, due to unrelated health complications, did not complete the course of the study.

Of the remaining seven subjects, all had normal red blood cell folate concentrations and were replete in vitamin B<sub>12</sub>. The cause of CRF in the seven subjects was hypertension (2), chronic glomerulonephritis (2), polycystic disease (1), reflux nephropathy (1) and obstructive uropathy (1). All had stable renal function as measured by plasma creatinine levels for three months before the trial began and they remained stable during the study.

The trial had a randomised, crossover, blinded study design, with the two treatments being 400 mg of DMG solution (Health World Limited, Queensland, Australia; Liquid preparation containing 50 mg/mL DMG in 20% ethanol by analysis) or placebo (20% ethanol) for 28 days. After 28 days, the subjects changed treatments without a wash-out period.

Fasting venous blood samples were drawn into EDTA tubes, and 12 hour urine samples were collected prior to treatment and at the end of treatment, on days 28 and 56. Blood samples were placed on ice until separated at 2000 *g* for 10 min, within an hour of collection. Urine volume was recorded and a 10 mL aliquot was taken for analysis. All samples were frozen at -20 °C until analysis.

Plasma homocysteine analysis was performed using standard clinical assays in Canterbury Health Laboratories. Plasma and urine glycine betaine, as well as plasma DMG, were measured by HPLC. The urine DMG was analysed using the <sup>1</sup>H-NMR assay described in this thesis, and as used for the rat and coffee study.<sup>40</sup> Plasma methionine was measured using a ninhydrin based amino

acid analyser. Urine creatinine was measured under standard clinical procedures based on the Jaffé reaction. Urine concentrations were converted to units of mmol/mol creatinine.

### 7.5.3 Results

There was no significant difference from baseline plasma glycine betaine ( $P = 0.452$ ), homocysteine ( $P = 0.624$ ) and methionine ( $P = 0.457$ ) after either the placebo or the DMG treatment. Plasma DMG concentration was significantly elevated after the DMG treatment, when compared to baseline ( $P = 0.021$ ) and the placebo had no effect on plasma DMG concentration ( $P = 0.997$ ) [Table 7.1].

Urine glycine betaine was not significantly altered after either of the treatments. Urine DMG was significantly elevated after the DMG treatment compared to the baseline ( $P = 0.014$ ) and the placebo ( $P = 0.008$ ). The placebo and baseline were not significantly different [Table 7.1].

	Baseline	DMG (mean $\pm$ SE)	Placebo
Plasma ( $\mu$ M)			
Homocysteine	17.7 $\pm$ 1.7	18.3 $\pm$ 1.3	17.9 $\pm$ 1.4
DMG	9.7 $\pm$ 1.1	71.9 $\pm$ 24.5 *	11.2 $\pm$ 1.7
Glycine betaine	40.0 $\pm$ 4.7	34.8 $\pm$ 3.0	37.0 $\pm$ 4.0
Methionine	27.8 $\pm$ 2.0	28.9 $\pm$ 2.1	26.6 $\pm$ 3.3
Creatinine	310 $\pm$ 40	330 $\pm$ 40	320 $\pm$ 40
Urine (mmol/mol creatinine)			
DMG	9.6 $\pm$ 3.0	34.5 $\pm$ 10.2 *	8.9 $\pm$ 2.1
Glycine betaine	79.6 $\pm$ 17.1	60.2 $\pm$ 13.5	66.7 $\pm$ 12.6

**Table 7.1** DMG trial results. Baseline, DMG treatment, and Placebo plasma and Urine concentrations for all the measured biochemicals. \* Significant difference to placebo and baseline ( $P < 0.05$ ).

### 7.5.4 Discussion

Plasma DMG concentrations were elevated in the patients at baseline and placebo, consistent with previous observations of CRF patients.<sup>41</sup> The normal range for DMG in plasma is

1.4-5.3  $\mu\text{M}$ .<sup>38</sup> Supplementation of 400 mg DMG over four weeks significantly elevated DMG in both the plasma and urine, but had no effect on glycine betaine, homocysteine, methionine or creatinine, compared with placebo.

Glycine betaine excretion was elevated in the baseline urine results, but this was unaffected by the supplementation with DMG. It was expected that plasma glycine betaine would increase, as a result of BHMT feedback inhibition by DMG, but this was not seen.

It is unlikely that these results would be any different in other population groups, where homeostasis of betaines is not disturbed as it is in CRF. It is possible that the two effects mentioned in the introduction, the inhibition of BHMT and the formation of mTHF are both occurring, and neutralising each other.

DMG supplementation in patients with CRF has no effect on plasma homocysteine or betaine homeostasis, resulting only in an increased plasma DMG and elevated DMG excretion.

## References

- (1) Brosnan, J. T.; Jacobs, R. L.; Stead, L. M.; Brosnan, M. E. *Acta Biochimica Polonica* **2004**, Methylation demand: A key determinant of homocysteine metabolism, *51*, 405-413.
- (2) Stead, L. M.; Jacobs, R. L.; Brosnan, M. E.; Brosnan, J. T. *Advances in Enzyme Regulation* **2004**, Methylation demand and homocysteine metabolism, *44*, 321-333.
- (3) Ulrey, C. L.; Liu, L.; Andrews, L. G.; Tollefsbol, T. O. *Human Molecular Genetics* **2005**, The impact of metabolism on DNA methylation, *14*, R139-R147.
- (4) Finkelstein, J. D.; Martin, J. J. *Journal of Biological Chemistry* **1986**, Methionine metabolism in mammals. Adaptation to methionine excess, *261*, 1582-1587.
- (5) Silberberg, J.; Crooks, R.; Wlodarczyk, J.; Nair, B.; Finucane, P.; Guo, X. W.; Xie, L. J.; Dudman, N. *Australian and New Zealand Journal of Medicine* **1997**, Reference range for % rise in homocysteine after oral methionine load, *27*, 717.
- (6) Miller, J. W.; Nadeau, M. R.; Smith, D.; Selhub, J. *American Journal of Clinical Nutrition* **1994**, Vitamin B-6 deficiency vs folate deficiency: comparison of responses to methionine loading in rats, *59*, 1033-1039.
- (7) Muller, T.; Woitalla, D.; Hauptmann, B.; Fowler, B.; Kuhn, W. *Neuroscience Letters* **2001**, Decrease of methionine and S-adenosylmethionine and increase of homocysteine in treated patients with Parkinson's disease, *308*, 54-56.
- (8) Stead, L. M.; Au, K. P.; Jacobs, R. L.; Brosnan, M. E.; Brosnan, J. T. *American Journal of Physiology. Endocrinology and Metabolism* **2001**, Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate, *281*, E1095-1100.
- (9) Olthof, M. R.; van Vliet, T.; Boelsma, E.; Verhoef, P. *Journal of Nutrition* **2003**, Low dose betaine supplementation leads to immediate and long term lowering of plasma homocysteine in healthy men and women, *133*, 4135-4138.
- (10) Olthof, M. R.; Verhoef, P. *Current Drug Metabolism* **2005**, Effects of betaine intake on plasma homocysteine concentrations and consequences for health, *6*, 15-22.
- (11) Singh, R. H.; Kruger, W. D.; Wang, L.; Pasquali, M.; Elsas, L. J., II *Genetics in Medicine* **2004**, Cystathionine b-synthase deficiency: effects of betaine supplementation after methionine restriction in B6-nonresponsive homocystinuria, *6*, 90-95.
- (12) Essen, A. v. In *Mathematics and Statistics*; University of Canterbury: Christchurch, New Zealand, 2003, p 34.

- (13) Dinning, J. S.; Neatrour, R.; Day, P. L. *Proceedings of the Society for Experimental Biology and Medicine* **1953**, Effect of diet on rat-liver betaine transmethylase, *84*, 499-501.
- (14) Ericson, L. E.; Harper, A. E. *Journal of Biological Chemistry* **1956**, Effect of diet on the betaine-homocysteine transmethylase activity of rat liver. I. Amino acids and protein, *219*, 49-58.
- (15) Finkelstein, J. D.; Kyle, W.; Harris, B. J. *Archives of Biochemistry and Biophysics* **1971**, Methionine metabolism in mammals. Regulation of homocysteine methyltransferases in rat tissue, *146*, 84-92.
- (16) Finkelstein, J. D.; Martin, J. J. *Journal of Biological Chemistry* **1984**, Methionine metabolism in mammals. Distribution of homocysteine between competing pathways, *259*, 9508-9513.
- (17) Wilcken, D. E. L.; Wilcken, B.; Dudman, N. P. B.; Tyrrell, P. A. *New England Journal of Medicine* **1983**, Homocystinuria-the effects of betaine in the treatment of patients not responsive to pyridoxine, *309*, 448-453.
- (18) McGregor, D. O.; Dellow, W. J.; Robson, R. A.; Lever, M.; George, P. M.; Chambers, S. T. *Kidney International* **2002**, Betaine supplementation decreases post-methionine hyperhomocysteinemia in chronic renal failure, *61*, 1040-1046.
- (19) Mulligan, J. D.; Laurie, T. J.; Garrow, T. A. *Journal of Nutritional Biochemistry* **1998**, An assay for betaine-homocysteine methyltransferase activity based on the microbiological detection of methionine, *9*, 351-354.
- (20) Mendum, M. L.; Smith, L. T. *Applied and Environmental Microbiology* **2002**, Characterization of glycine betaine porter I from *Listeria monocytogenes* and its roles in salt and chill tolerance, *68*, 813-819.
- (21) Goeger, D. E.; Ganther, H. E. *Archives of Biochemistry and Biophysics* **1993**, Homocysteine-dependent demethylation of trimethylselenonium ion and selenobetaine with methionine formation., *302*, 222-227.
- (22) Lever, M.; Bason, L.; Leaver, C.; Hayman, C. M.; Chambers, S. T. *Analytical Biochemistry* **1992**, Same-day batch measurement of glycine betaine, carnitine, and other betaines in biological material, *205*, 14-21.
- (23) De Oliveira, A. C.; D'Almeida, V.; Hipolide, D. C.; Nobrega, J. N.; Tufik, S. *Canadian Journal of Physiology and Pharmacology* **2002**, Sleep deprivation reduces total plasma homocysteine levels in rats, *80*, 193-197.

- (24) Lever, M.; Sizeland, P. C. B.; Bason, L. M.; Hayman, C. M.; Chambers, S. T. *Biochimica et Biophysica Acta* **1994**, Glycine betaine and proline betaine in human blood and urine, *1200*, 259-264.
- (25) Bostom, A. G.; Lathrop, L. *Kidney International* **1997**, Hyperhomocysteinemia in end-stage renal disease: Prevalence, etiology, and potential relationship to arteriosclerotic outcomes, *52*, 10-20.
- (26) Ericson, L. E. *Acta Chemica Scandinavica* **1960**, Betaine-homocysteine methyltransferases. I. Distribution in nature, *14*, 2102-2112.
- (27) Maw, G. A. *Biochemical Journal* **1959**, Thetin-homocysteine transmethylase. The distribution of the enzyme, studied with the aid of trimethylsulfonium chloride as substrate, *72*, 602-608.
- (28) McKeever, M. P.; Weir, D. G.; Molloy, A.; Scott, J. M. *Clinical Science* **1991**, Betaine-homocysteine methyltransferase: organ distribution in man, pig and rat and subcellular distribution in the rat, *81*, 551-556.
- (29) Xue, G. P.; Snoswell, A. M. *Comparative Biochemistry and Physiology, Part B: Biochemistry & Molecular Biology* **1986**, Developmental changes in the activities of enzymes related to methyl group metabolism in sheep tissues, *83B*, 115-120.
- (30) Stolzenberg-Solomon, R. Z.; Miller, E. R., III; Maguire, M. G.; Selhub, J.; Appel, L. J. *American Journal of Clinical Nutrition* **1999**, Association of dietary protein intake and coffee consumption with serum homocysteine concentrations in an older population, *69*, 467-475.
- (31) Nygård, O.; Refsum, H.; Ueland, P. M.; Vollset, S. E. *American Journal of Clinical Nutrition* **1998**, Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland homocysteine study, *67*, 263-270.
- (32) Urgert, R.; van Vliet, T.; Zock, P. L.; Katan, M. B. *American Journal of Clinical Nutrition* **2000**, Heavy coffee consumption and plasma homocysteine: a randomized controlled trial in healthy volunteers, *72*, 1107-1110.
- (33) Grubben, M. J.; Boers, G. H.; Blom, H. J.; Broekhuizen, R.; de Jong, R.; van Rijt, L.; de Ruijter, E.; Swinkels, D. W.; Nagengast, F. M.; Katan, M. B. *American Journal of Clinical Nutrition* **2000**, Unfiltered coffee increases plasma homocysteine concentrations in healthy volunteers: a randomized trial, *71*, 480-484.

- (34) Christensen, B.; Mosdol, A.; Retterstol, L.; Landaas, S.; Thelle, D. S. *American Journal of Clinical Nutrition* **2001**, Abstention from filtered coffee reduces the concentrations of plasma homocysteine and serum cholesterol--a randomized controlled trial, *74*, 302-307.
- (35) de Zwart, F. J.; Slow, S.; Payne, R. J.; Lever, M.; George, P. M.; Gerrard, J. A.; Chambers, S. T. *Food Chemistry* **2003**, Glycine betaine and glycine betaine analogues in common foods, *83*, 197-204.
- (36) Verhoef, P.; Pasman, W. J.; Van Vliet, T.; Urgert, R.; Katan, M. B. *American Journal of Clinical Nutrition* **2002**, Contribution of caffeine to the homocysteine-raising effect of coffee: a randomized controlled trial in humans, *76*, 1244-1248.
- (37) Olthof, M. R.; Hollman, P. C.; Zock, P. L.; Katan, M. B. *American Journal of Clinical Nutrition* **2001**, Consumption of high doses of chlorogenic acid, present in coffee, or of black tea increases plasma total homocysteine concentrations in humans, *73*, 532-538.
- (38) Allen, R. H.; Stabler, S. P.; Lindenbaum, J. *Metabolism: Clinical and Experimental* **1993**, Serum betaine, N,N-dimethylglycine and N-methylglycine levels in patients with cobalamin and folate deficiency and related inborn errors of metabolism, *42*, 1448-1460.
- (39) Graber, C. D.; Goust, J. M.; Glassman, A. D.; Kendall, R.; Loadholt, C. B. *Journal of Infectious Diseases* **1981**, Immunomodulating properties of dimethylglycine in humans, *143*, 101-105.
- (40) Slow, S.; Lever, M.; Lee, M. B.; George, P. M.; Chambers, S. T. *International Journal of Biochemistry & Cell Biology* **2004**, Betaine analogues alter homocysteine metabolism in rats, *36*, 870-880.
- (41) McGregor, D. O.; Dellow, W. J.; Lever, M.; George, P. M.; Robson, R. A.; Chambers, S. T. *Kidney International* **2001**, Dimethylglycine accumulates in uremia and predicts elevated plasma homocysteine concentrations, *59*, 2267-2272.

## Chapter 8

# Conclusions and Future Work



## CHAPTER 8: Conclusions and Future Work

### 8.1 Thesis Aims

Most of the aims of this thesis involved the application of  $^1\text{H}$ -NMR spectroscopy to biological samples. Specifically the aims were:

1. To develop a method for the purification of BHMT from liver samples [Chapter 2].
2. To develop a  $^1\text{H}$ -NMR spectroscopic assay for BHMT for the purposes of detection of BHMT, and the measurement of kinetic constants for BHMT [Chapters 3 and 4].
3. To use  $^1\text{H}$ -NMR spectroscopy to investigate the substrate specificity of BHMT from rat liver [Chapter 5].
4. To make observations of BHMT from alternative sources, including Agnathan liver samples, and to partially characterise the enzyme for comparison with rat BHMT [Chapter 6].
5. To use  $^1\text{H}$ -NMR spectroscopy to measure methylamines and related compounds in urine as an indirect method for the detection of BHMT activity *in vivo* [Chapter 7].

### 8.2 Reproducible BHMT Purification

In Chapter 2, the purification of BHMT from rat liver was described, using several elements of previously reported preparations. In short, purification was based on tissue homogenisation, followed by heat treatment and then size exclusion chromatography. The methods were further developed and it was found that BHMT can survive at higher temperatures, although for much shorter times than had previously been described. This enabled the initial purification step to be greatly improved from a ~1.5 fold purification (70 °C for 5 min) step to a ~5 fold one (90 °C for 2 min). These steps were used in all rat liver extractions. The purified BHMT was not homogeneous,

but SDS-PAGE analysis showed considerable enrichment of BHMT activity. It was estimated by visual inspection of SDS-PAGE results that BHMT accounted for approximately 80 % of the total protein in the final product.

Further work to determine the BHMT concentration could be performed and used to “correct” the concentration of active BHMT in the kinetic calculations. For example, an ELISA technique, based on antibodies raised against rat liver BHMT, would be a suitable method for analysis of total BHMT concentration. Active site titration, to determine the concentration of active BHMT in the solution, might also be worth doing.<sup>1</sup> This procedure uses an interaction between a known competitive inhibitor of the enzyme and some method of detecting its interaction with the protein. The method may have to be adapted to include kinetic techniques, as described by Brocklehurst *et al.*<sup>1</sup> A suitable inhibitor might be *S*-( $\gamma$ -carboxybutyl)-L-homocysteine (CBH) which was described in **Chapter 1**. Brocklehurst *et al.* related the number of active sites with the catalytic activity of the enzyme, showing that a mathematical relationship can be drawn between the two and that this can be used to determine the number of active sites present in the solution. The application of their analysis to the BHMT extracts should afford an estimate of the active BHMT concentration. It is also possible that the boronic acid betaine developed by Lee *et al.* may be useful in these studies.<sup>2</sup> Zinc measurements may also be conducted and used to estimate the concentration of BHMT in solution, however, this would require the use of zinc-free solutions throughout the purification of BHMT.

Anion exchange chromatography of the rat extract gave a solution in which the BHMT was initially active, however, that activity was reduced to undetectable levels after approximately 48-60 h. This is consistent with a previous report that BHMT from Sprague-Dawley rats was labile when purified.<sup>2</sup> This group also obtained a protein comprised of 45 kDa subunits and having a total mass of ~260-270 kDa. This is consistent with hexameric BHMT, which they purified to near homogeneity. They found that the BHMT activity was labile unless dimethylglycine and

homocysteine were present in the solution. Both of these results imply that there is a stabilising factor present in impure BHMT solutions which is not removed in the procedures described in **Chapter 2**. It is also possible that there is some inhibitor which slowly leaches out of the DEAE Sephadex, removing BHMT activity: although it is not clear why this would lead to a slow loss of activity.

The quaternary structure of liver BHMT is subject to an ongoing debate, with recombinant human BHMT having been shown to be a tetramer of 45 kDa subunits by X-ray crystal structure analysis.<sup>3-5</sup> Analysis of most liver extracts have estimated the native enzyme to be larger, approximately 250-270 kDa in size, being consistent with a hexameric structure. The results presented in **Chapter 2** are consistent with a hexameric structure, although the measurements indicated a size slightly smaller than expected. Further work needs to be done to explain the differences observed. It is possible that the naturally occurring BHMT is a hexamer, but that the recombinant protein is not. Some post-translational modification may occur in the mammalian liver. Analytical ultracentrifugation may allow the elucidation of the true quaternary structure of BHMT. Ultracentrifugation accompanied with mass spectrometry may also show any glycosylation which may occur on the protein. One simple first experiment should include the same gel filtration analysis exploring the quaternary structure of recombinant and liver BHMT. This should reveal any differences between the two proteins.

The kinetic and specificity results obtained in this thesis are in good agreement with other measurements made internationally, implying that the protein extracted was of a similar nature to those obtained by other groups.<sup>2,6-10</sup> Furthermore, the same purification procedures were employed on several occasions during the progress of the research presented in this thesis, and always gave similar purification when visualised with SDS-PAGE analysis.

*N*-terminal sequence analysis revealed a variable *N*-terminal sequence suggesting that amino-peptidase had modified the BHMT during preparation. Protease inhibitors have been used

in the BHMT purification by some research groups,<sup>2</sup> and it may be possible to improve the yield and integrity of active BHMT by adding protease inhibitors to the buffers used in the procedures developed in this thesis. This would need care as EDTA, commonly used as a metal-protease inhibitor, could chelate the zinc cation from the active site of BHMT, particularly if it was present during the high temperatures involved in the denaturing of the homogenate. It is unknown if the amino-peptidase activity observed is a consequence of liver enzymes, or bacterial proteases introduced after the heat denaturation step.

### 8.3 <sup>1</sup>H-NMR Spectroscopic Assay for BHMT and Kinetic Measurements

Once BHMT had been reproducibly purified, work proceeded on the development of a <sup>1</sup>H-NMR assays for physiological betaines and BHMT activity. Spin lattice relaxation constants were determined for glycine betaine, DMG and methionine in water, and appropriate delays were then built into the basic pulse sequences. Water suppression with pulsed field gradients (DPFGSE) and presaturation of the water resonance were also explored, and for kinetic measurements, DPGFSE was used to ensure adequate suppression of water. The assay was based on integrations of the <sup>1</sup>H-NMR singlet resonances of glycine betaine, DMG and methionine (3.22, 2.86 and 2.10 ppm respectively) all compared to the *tert*-butanol signal (1.15 ppm). Standard lines of glycine betaine, DMG and methionine were determined with respect to *tert*-butanol, allowing quantification of each reaction component during the assay. Plots of the concentration of DMG over time were used to determine the rate of methyl transfer. The reaction was modelled on the Michaelis Menten steady-state equation, and the  $K_m$  for glycine betaine was determined to be  $0.19 \pm 0.03$  mM, and  $V_{max}$  for BHMT was  $17 \pm 0.7$  nmol min<sup>-1</sup> mg<sup>-1</sup>, converting to a turnover number for each active site of  $\sim 0.8$  min<sup>-1</sup>. These results are in good agreement with previous estimates of  $K_m$  and  $V_{max}$  for the rat liver enzyme.<sup>2,11</sup>

#### 8.4 Methyl Donor Substrate Specificity

The  $^1\text{H}$ -NMR spectroscopic assay developed in **Chapter 3** allowed, for the first time, direct monitoring of methyl transfer from a range of potential substrates. The work of Ericson from 1960 was reviewed and investigated, and several new betaines were tested for activity and inhibition against rat liver BHMT.<sup>12</sup> It was found that BHMT activity is affected by chain length, methylated heteroatom and  $\alpha$ -substitution in the donor betaine, but that DMT, DMSP, trimethylsulfonium ions, selenobetaine and DMSeP can all act as methyl donors in BHMT. Co-incubation of glycine betaine and betaine analogues did not provide clear evidence of inhibition. These findings were in contrast to the work performed by Ericson and Mulligan *et al*/ who found BHMT activity with several alternative betaine sources, including proline betaine, via the colorimetric and microbiological assays, respectively.<sup>12,13</sup>

The results presented by Ericson and Mulligan may be a result of problems with the colorimetric and microbiological assays. The nitroprusside dye used in this assay might react with some of the betaine analogues, leading to positive readings when no methionine is being produced. This may lead to false positive readings in the BHMT assay, as the nitroprusside that they used may give a colour change without the production of methionine. Similarly, the microbiological assay may not be linear at low levels of activity, with a small concentration of methionine giving a rapid growth in *E. coli*.

Molecular modelling of the chemical reaction thermodynamics was performed for some of the more active betaines, including glycine betaine, propiobetaine, DMT, DMSP, selenobetaine and the tetramethylammonium and trimethylsulfonium ions to elucidate any thermodynamic reasons for the differences in BHMT activity seen. It was shown that at higher levels of computational modelling, thermodynamic differences between the ammonium and sulfonium analogues could be seen, partially explaining the much higher activities of the sulfonium analogues. Similar results were seen with the sulfonio and selenonio analogues. These results, however, all support the

experimental result that the sulfonio and seleno-betaines are much faster methyl donors in BHMT than the amino betaines. The modelling also verified that there was a thermodynamic reason for the loss of activity from glycine betaine (nitrogen heteroatom) to phosphonio and arseno-betaine (phosphorus and arseno- heteroatoms, respectively). This is likely to be due to the effect of empty d orbitals, which phosphorus and arsenic both have at their disposal.

The lack of activity seen with the  $\alpha$ -substituted betaines, the betaines with increased chain length, and the naturally occurring betaines is likely to be due to a steric effect, where interactions between the betaine and critical residues in BHMT have either been disrupted, or the larger size of the betaine prevents access to the active site of BHMT. There was, however, potentially a small allosteric effect observed with phenylalanine betaine, and with ectoine, which led to enhanced methyl transfer when they were co-incubated with glycine betaine. This may be a consequence of conformational changes in BHMT as the phenylalanine betaine and ectoine bind to the active site, inducing a greater catalytic efficiency at the other active sites in the active BHMT protein.

Recent work which has elucidated the crystal structure of BHMT will provide the next advances in this work. Molecular modelling of the active site of BHMT, with the analogues identified here, should provide a much greater understanding of how BHMT achieves methylation, and the catalytic rate seen in the enzyme. Furthermore, NMR spectroscopic elucidation of the solution state structure may be employed to explore the binding of betaines to the known betaine binding amino-acid residues in the BHMT active site.<sup>5,14</sup> There are several experiments that are available to the NMR researcher, notably diffusion experiments,<sup>15</sup> which may be able to add to the IF experiments to further expand and explain the role of betaine analogues in the BHMT reaction.

### 8.5 Comparison of Agnathan and Mammalian Liver BHMT

The distribution of BHMT in nature poses an interesting study. The methionine-homocysteine cycle is of vital importance to basic biochemistry. It has been shown that the known

mammalian forms of BHMT are all remarkably similar, sharing a high degree of gene and amino acid identity [Figure 6.1]. This evidence, including the discovery of BHMT activity in agnathan liver, implies a common ancestral enzyme.

*N*-terminal sequencing of rat protein conclusively revealed the presence of the expected BHMT subunit in the active rat fractions. No sequence could be obtained from the hagfish samples. The likely cause of this is acetylation on the *N*-terminus of the hagfish BHMT. *In situ* acid/alcohol washing of the hagfish PVDF did not yield any *N*-terminal sequence data. Further work is required to improve the purification of BHMT from the hagfish and to resolve the protein sequence to allow evolutionary comparison. Cyanogen bromide cleavage of the 45 kDa PVDF band which has already been isolated might further afford information on the structure of the protein seen. Cyanogen bromide cleaves peptides at methionine residues, and (using rat BHMT as a model) would be expected to give 9 shorter polypeptides, one of which will remain *N*-terminally blocked. If this work is done in solution, preparative HPLC analysis may be able to resolve and isolate these fragments for *N*-terminal sequencing or mass spectroscopic analysis.

Hagfish and lamprey liver cDNA libraries were prepared by Dr Victoria Metcalf working with the group. These may be employed to further isolate a BHMT cDNA from the hagfish liver. One approach would be to use functional complementation, using *E. coli* J5-3, a methionine auxotroph. Here the J5-3 would be transformed with a cDNA plasmid library, and clones coding for a functional BHMT activity can be selected for sequencing which will lead to the identification of the active BHMT.<sup>13</sup>

The hagfish BHMT was shown to have similar substrate specificity to the rat liver BHMT, indicating that it potentially shares a common betaine and homocysteine binding motif. These studies can be expanded to include the libraries of betaines investigated in Chapter 5 here in mammalian liver BHMT, as well as others, including trimethyltelluride and trimethylselenonium ions.

The investigations on Agnathan BHMT should also be expanded to include more distant animals, potentially the swan-mussel, in which BHMT activity was identified during Ericson's investigations.<sup>16</sup> Furthermore, BHMT activity has been identified in plants, bacteria and fungi.<sup>17-20</sup> The investigations of the acetone powders presented in **Chapter 6**, imply that there is either no BHMT in lemon shark livers, or that it is not stable as an acetone powder. Fresh homogenates of shark livers should be prepared and assayed for BHMT activity, to elucidate the absence or presence of BHMT in shark livers. Also, purification of BHMT activity from these livers should be refined to give a higher purity than what was obtained in this work.

### 8.6 <sup>1</sup>H-NMR Spectroscopic Assay for Methylamines in Urine

The NMR-based assay for glycine betaine used in the kinetic assay was expanded and verified to allow clinical assays of various betaines in urine. Clinically, there are several methylamines which are useful indicators of disease. These include creatine, creatinine, trimethylamine, trimethylamine *N*-oxide, proline betaine, trigonelline and dimethylglycine.<sup>21-29</sup> NMR spectroscopy has been used to measure each of these metabolites before, but there has never been a validation process published, verifying the suitability of NMR spectroscopy for clinical studies.

The within and inter-batch coefficients of variance for each of the metabolites listed above were below 7.5 % and 8.5 % respectively, with limits of detection (*s/n* >3) of 15-25 μM. Previous research into the measurement of small molecules in urine has employed complicated chemical manipulation to help remove water signals, often dehydrating or freeze drying the samples, then replacing the solvent with D<sub>2</sub>O, providing an internal lock standard. It was found that presaturation of the water resonance for 3 s prior to the 90° pulse and acquisition was sufficient to remove the water signal for quantifiable results. Carnitine was also identified in the spectrum, and was not co-resonant with any of the metabolites measured.



Metabonomics/Metabolomics is a rapidly developing field, being driven by developments in high-field NMR spectroscopy. Metabolomic analyses involve running 'fingerprint' spectra, with rapid throughput, and using automated computerised systems to analysis and diagnose metabolic disorders from those spectra. The NMR spectroscopic techniques developed here were labour intensive, but can be automated. Implementation of the metabolomic software being developed by Varian and Bruker will allow much faster processing and analysis. Current HPLC techniques in use at Canterbury Health Laboratories for the measurement of methylamines involve 60 min run-times and large volumes of acetonitrile. The NMR spectroscopic assay takes less than 2 min, and is capable of discerning DMG and TMA/TMAO, both of which are difficult to detect, resolve and quantify by HPLC. The versatility of the NMR assay was demonstrated during the rat experiment [Chapter 7] and the rapid adaptation of the currently existent assay to measure the sulfoxide of the DMT demethylation reaction.

The potential exists to expand this work to encompass a much larger range of metabolites. There is precedent for a number of metabolites to be analysed via NMR spectroscopy, including citrate, alanine and glucose, amongst numerous other metabolites.<sup>30-32</sup> There are many other potential metabolites which could be measured using <sup>1</sup>H-NMR spectroscopy, including acetyl-carnitine and carnitine. As metabolomics develops, along with high-field NMR spectrometers, the list of metabolites measurable should grow to make NMR spectroscopy a valuable tool in diagnosis.

## 8.7 Future Experiments

There are four experiments which could be conducted initially to clarify some of the results observed in this thesis.

1. Protein sequencing of hagfish BHMT should identify the protein responsible for BHMT activity. Better purification is likely to be needed, but if *N*-terminal blocking remains a problem, cyanogen bromide cleavage, followed by reverse-phase HPLC and collection of the fragments should help identify the protein.
2. Kinetic analysis of DMT and DMSP in the presence of DMG should determine the  $K_i$  for DMG.
3. Incubation of hagfish BHMT in the presence of a wider array of betaine analogues should be conducted to better compare the two enzymes.
4. Carnitine was identified in the urine  $^1\text{H}$ -NMR spectroscopic assay, but not used in any of the validation work. Carnitine would be useful for clinical work at Canterbury Health Laboratories, so the validation work outlined in **Chapter 4** could be repeated with carnitine.

## 8.8 Conclusion

There is little question about the necessity of research into homocysteine and CVD. Despite recent reports which found that although plasma homocysteine concentration is reduced after supplementation with folate and vitamin B<sub>12</sub>, there was no associated reduction in further CVD risk (with the exception of stroke, the risk of which was reduced by their intervention). These studies have all ignored the potential for BHMT to also reduce plasma homocysteine, so there is still potential for BHMT to be explored for CVD therapeutics. Infact, BHMT might be a more important junction than MS, because the link between homocysteine and CVD is well described, yet folate treatment does not appear to protect against a second atherosclerotic events (although the risk of stroke is reduced by this intervention).

However, the interaction between glycine betaine and homocysteine is not yet fully elucidated. There is only one known enzyme which can demethylate glycine betaine and remove homocysteine to form methionine (BHMT), but there are several betaine analogues, and other naturally occurring chemicals which may affect BHMT activity *in vivo*, which have not been explored. The NMR spectroscopic assay is the best technique for the exploration of these effects, because it offers a continuous method for monitoring the reaction, is applicable for all potential analogues and is a direct measure of the reaction, unlike some of the microbiological assays. Furthermore, NMR spectroscopy also allows for further investigation of the binding of betaine analogues using diffusion measurements. NMR spectroscopy may also be used to solve the solution-state structure of the enzyme, which should help in the exploration of betaine/homocysteine relationships *in vivo*.

## References

- (1) Brocklehurst, K.; Resmini, M.; Topham, C. M. *Methods* **2001**, Kinetic and Titration Methods for Determination of Active Site Contents of Enzyme and Catalytic Antibody Preparations., *24*, 153-167.
- (2) Lee, K. H.; Cava, M.; Amiri, P.; Ottoboni, T.; Lindquist, R. N. *Archives of Biochemistry and Biophysics* **1992**, Betaine:homocysteine methyltransferase from rat liver: purification and inhibition by a boronic acid substrate analog, *292*, 77-86.
- (3) Bose, N.; Momany, C. *Acta Crystallographica, Section D: Biological Crystallography* **2001**, Crystallization and preliminary x-ray crystallographic studies of recombinant human betaine-homocysteine S-methyltransferase., *D57*, 431-433.
- (4) Evans, J. C.; Huddler, D. P.; Jiracek, J.; Castro, C.; Millian, N. S.; Garrow, T. A.; Ludwig, M. L. *Structure* **2002**, Betaine-Homocysteine Methyltransferase Zinc in a Distorted Barrel, *10*, 1159-1171.
- (5) Gonzalez, B.; Pajares, M. A.; Martinez-Ripoll, M.; Blundell, T. L.; Sanz-Aparicio, J. *Journal of Molecular Biology* **2004**, Crystal Structure of Rat Liver Betaine Homocysteine S-Methyltransferase Reveals New Oligomerization Features and Conformational Changes Upon Substrate Binding, *338*, 771-782.
- (6) Collinsova, M.; Castro, C.; Garrow, T. A.; Yiotakis, A.; Dive, V.; Jiracek, J. *Chemistry & Biology* **2003**, Combining Combinatorial Chemistry and Affinity Chromatography. Highly Selective Inhibitors of Human Betaine: Homocysteine S-Methyltransferase, *10*, 113-122.
- (7) Garrow, T. A. *Journal of Biological Chemistry* **1996**, Purification, kinetic properties, and cDNA cloning of mammalian betaine-homocysteine methyltransferase, *271*, 22831-22838.
- (8) Lee, M. B.; Blunt, J. W.; Lever, M.; George, P. M. *Analytical Biochemistry* **2004**, A nuclear-magnetic-resonance-based assay for betaine-homocysteine methyltransferase activity, *330*, 199-205.
- (9) Millian, N. S.; Garrow, T. A. *Archives of Biochemistry and Biophysics* **1998**, Human betaine-homocysteine methyltransferase is a zinc metalloenzyme, *356*, 93-98.
- (10) Sunden, S. L. F.; Renduchintala, M. S.; Park, E. I.; Miklasz, S. D.; Garrow, T. A. *Archives of Biochemistry and Biophysics* **1997**, Betaine-homocysteine methyltransferase expression in porcine and human tissues and chromosomal localization of the human gene, *345*, 171-174.

- (11) Finkelstein, J. D.; Harris, B. J.; Kyle, W. E. *Archives of Biochemistry and Biophysics* **1972**, Methionine metabolism in mammals: kinetic study of betaine-homocysteine methyltransferase., *153*, 320-324.
- (12) Ericson, L. E. *Acta Chemica Scandinavica* **1960**, Betaine-homocysteine methyltransferases. III. The methyl-donor specificity of the transferase isolated from pig liver, *14*, 2127-2134.
- (13) Mulligan, J. D.; Laurie, T. J.; Garrow, T. A. *Journal of Nutritional Biochemistry* **1998**, An assay for betaine-homocysteine methyltransferase activity based on the microbiological detection of methionine, *9*, 351-354.
- (14) Szegedi, S. S.; Garrow, T. A. *Archives of Biochemistry and Biophysics* **2004**, Oligomerization is required for betaine-homocysteine S-methyltransferase function, *426*, 32–42.
- (15) Lucas, L. H.; Larive, C. K. *Concepts in Magnetic Resonance, Part A* **2004**, Measuring ligand-protein binding using NMR diffusion experiments, *20A*, 24-41.
- (16) Ericson, L. E. *Acta Chemica Scandinavica* **1960**, Betaine-homocysteine methyltransferases. I. Distribution in nature, *14*, 2102-2112.
- (17) Baburina, O. K.; Shevyakova, N. I. *Russian Journal of Plant Physiology (Translation of Fiziologiya Rastanii (Moscow))* **1995**, Betaines as methyl-group donors in alfalfa shoots exposed to saline media, *42*, 764-771.
- (18) Rodionov, D. A.; Vitreschak, A. G.; Mironov, A. A.; Gelfand, M. S. *Nucleic Acids Research* **2004**, Comparative genomics of the methionine metabolism in Gram-positive bacteria: a variety of regulatory systems, *32*, 3340-3353.
- (19) Incharoensakdi, A.; Waditee, R. *Current Microbiology* **2000**, Degradation of glycinebetaine by betaine-homocysteine methyltransferase in *Aphanothece halophytica*: effect of salt downshock and starvation., *41*, 227-231.
- (20) Pichereau, V.; Pocard, J.-A.; Hamelin, J.; Blanco, C.; Bernard, T. *Applied and Environmental Microbiology* **1998**, Differential effects of dimethylsulfoniopropionate, dimethylsulfonioacetate, and other S-methylated compounds on the growth of *Sinorhizobium meliloti* at low and high osmolarities, *64*, 1420-1429.
- (21) Lee, M. B.; Storer, M. K.; Blunt, J. W.; Lever, M. *Clinica Chimica Acta* **2006**, Validation of <sup>1</sup>H NMR spectroscopy as an analytical tool for methylamine metabolites in urine, *365*, 264-269.
- (22) Olthof, M. R.; Verhoef, P. *Current Drug Metabolism* **2005**, Effects of betaine intake on plasma homocysteine concentrations and consequences for health, *6*, 15-22.

- (23) Finkelstein, J.; Martin, J. *J. Biol. Chem.* **1984**, Methionine metabolism in mammals. Distribution of homocysteine between competing pathways, *259*, 9508-9513.
- (24) Craig, S. A. S. *American Journal of Clinical Nutrition* **2004**, Betaine in human nutrition, *80*, 539-549.
- (25) Slow, S.; Lever, M.; Lee, M. B.; George, P. M.; Chambers, S. T. *International Journal of Biochemistry and Cell Biology* **2004**, Betaine analogues alter homocysteine metabolism in rats, *36*, 870-880.
- (26) Melse-Boonstra, A.; Holm, P. I.; Ueland, P. M.; Olthof, M.; Clarke, R.; Verhoef, P. *Am J Clin Nutr* **2005**, Betaine concentration as a determinant of fasting total homocysteine concentrations and the effect of folic acid supplementation on betaine concentrations, *81*, 1378-1382.
- (27) Lever, M.; George, P. M.; Dellow, W. J.; Scott, R. S.; Chambers, S. T. *Metabolism-Clinical and Experimental* **2005**, Homocysteine, glycine betaine, and N,N-dimethylglycine in patients attending a lipid clinic, *54*, 1-14.
- (28) Mitchell, S. C.; Smith, R. L. *Drug Metabolism and Disposition* **2001**, Trimethylaminuria: the fish malodor syndrome, *29*, 517-521.
- (29) Maschke, S.; Wahl, W.; Azaroual, N.; Boulet, O.; Crunelle, V.; Imbenotte, M.; Foulard, M.; Vermeersch, G.; Lhermitte, M. *Clinica Chimica Acta* **1997**, <sup>1</sup>H NMR analysis of trimethylamine in urine for the diagnosis of fish-odour syndrome, *263*, 139-146.
- (30) Bales, J. R.; Higham, D. P.; Howe, I.; Nicholson, J. K.; Sadler, P. J. *Clinical Chemistry* **1984**, Use of high-resolution proton nuclear magnetic resonance spectroscopy for rapid multi-component analysis of urine, *30*, 426-432.
- (31) Fulton, D. B.; Sayer, B. G.; Bain, A. D.; Malle, H. V. *Analytical Chemistry* **1992**, Detection and determination of dilute, low molecular weight organic compounds in water by 500 MHz proton nuclear magnetic resonance spectroscopy., *64*, 349-353.
- (32) Nicholson, J. K.; O'Flynn, M. P.; Sadler, P. J.; Macleod, A. F.; Juul, S. M.; Sonksen, P. H. *Biochemical Journal* **1984**, Proton-nuclear-magnetic-resonance studies of serum, plasma and urine from fasting normal and diabetic subjects, *217*, 365-375.

## Appendices

## Appendix 1 Tryptic Digest of BHMT

Complete table of Tryptic digest fragments from rat liver BHMT including predicted mass spectral peaks.

Fragment	Fragment Sequence	Mass (Daltons)	M+1	M+2	M+3	M+4	M+5
T1	APIAGK	555.34	556.35	278.68	186.12	139.84	112.08
T2	K	146.11	147.11	74.06	49.71	37.53	30.23
T3	AK	217.14	218.15	109.58	73.39	55.29	44.44
T4	R	174.11	175.12	88.06	59.05	44.54	35.83
T5	GILER	586.34	587.35	294.18	196.46	147.59	118.28
	LNAGEVVIGDGGFVFALE						
T6	K	1934.01	1935.02	968.02	645.68	484.51	387.81
T7	R	174.11	175.12	88.06	59.05	44.54	35.83
T8	GYVK	465.26	466.27	233.64	156.09	117.32	94.06
T9	AGPWTPEAAVEHPEAVR	1815.89	1816.9	908.95	606.3	454.98	364.19
T10	QLHR	552.31	553.32	277.16	185.11	139.09	111.47
T11	EFLR	563.31	564.31	282.66	188.78	141.83	113.67
T12	AGSNVMQTFTFYASEDK	1894.84	1895.85	948.43	632.62	474.72	379.98
T13	LENR	530.28	531.29	266.15	177.77	133.58	107.06
T14	GNYYAEK	779.38	780.39	390.7	260.8	195.85	156.88
T15	ISGQK	531.3	532.31	266.66	178.11	133.83	107.27
T16	VNEAAJDIAR	1117.52	1118.53	559.77	373.51	280.39	224.51
	QVADEGDALVAGGVST						
T17	PSYLSJK	2451.16	2452.17	1226.59	818.06	613.8	491.24
T18	SETEVK	691.34	692.35	346.68	231.45	173.84	139.28
T19	K	146.11	147.11	74.06	49.71	37.53	30.23
T20	IFHQQLVFMK	1418.74	1419.75	710.38	473.92	355.69	284.76
T21	K	146.11	147.11	74.06	49.71	37.53	30.23
	NVDFLIAEYFEHVVEEAVW						
T22	AVEALK	2820.4	2821.41	1411.21	941.14	706.11	565.09
	TSGKPIAATMJIGPEGDLH						
T23	GVSPGEJAVR	2966.4	2967.41	1484.21	989.81	742.61	594.29
T24	LVK	358.26	359.27	180.14	120.43	90.57	72.66
	AGAAIVGVNJHFDPTSL						
T25	QTIK	2285.15	2286.16	1143.58	762.72	572.29	458.04
T26	LMK	390.23	391.24	196.12	131.08	98.57	79.05
T27	EGLEAAR	744.38	745.38	373.2	249.13	187.1	149.88
T28	LK	259.19	260.2	130.6	87.4	65.81	52.85
T29	AYLMSHALAYHTPDJGK	1933.88	1934.89	967.95	645.63	484.48	387.78
T30	QGFDLPEFPFGLPR	1860.94	1861.95	931.48	621.32	466.24	373.2
T31	VATR	445.26	446.27	223.64	149.43	112.32	90.06
T32	WDIQK	688.35	689.36	345.19	230.46	173.1	138.68



# Appendix 1 – Tryptic Digest of BHMT

T33	YAR	408.21	409.22	205.11	137.08	103.06	82.65
T34	EAYNLGVR	920.47	921.48	461.24	307.83	231.13	185.1
T35	YIGGJJGFEPYHIR	1727.75	1728.76	864.89	576.93	432.95	346.56
T36	AIAEELAPER	1097.57	1098.58	549.79	366.87	275.4	220.52
T37	GFLPPASEK	944.5	945.5	473.26	315.84	237.13	189.91
T38	HGSWGSGLDMHTKPWIR	1963.95	1964.96	982.98	655.66	491.99	393.8
T39	AR	245.15	246.16	123.58	82.72	62.3	50.04
T40	AR	245.15	246.16	123.58	82.72	62.3	50.04
T41	K	146.11	147.11	74.06	49.71	37.53	30.23
T42	EYWQNLR	1007.48	1008.49	504.75	336.84	252.88	202.5
	IASGRRPYNPSMSKPD						
T43	WGVTK	2261.13	2262.13	1131.57	754.72	566.29	453.23
T44	GAAELMQQK	974.49	975.49	488.25	325.84	244.63	195.9
T45	EATTEQQLR	1074.53	1075.54	538.27	359.18	269.64	215.91
T46	ALFEK	606.34	607.35	304.18	203.12	152.59	122.28
T47	QK	274.16	275.17	138.09	92.4	69.55	55.84
T48	FK	293.17	294.18	147.59	98.73	74.3	59.64
T49	SAQ	304.14	305.15	153.08	102.39	77.04	61.84